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Immunologic Aspects of Cancer: *A Review**

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The field of tumor immunity, when last surveyed (206), was characterized by a preoccupation with induced resistance and diagnostic serology. During the ensuing decade, emphasis has shifted toward problems of tissue specificity and a more basic concern with the genetic control and chemical nature of cellular antigens. Improvements in centrifugal and enzymatic fractionation and purification procedures, isotopic labeling of gamma globulins, and synthesis of protein-carcinogen conjugates have opened up new possibilities for immunochemical investigations. Meanwhile, the biologic attack on the question of distinctive tumor constituents has profited from advances in immunogenetics and virology.

Experimental design in general appears to have been influenced by Woglom's (219) sobering admonitions and is today guided by a more critical awareness of genetic and serologic pitfalls. Although the outlook toward clinical application of new facts is no less dim than it was 10 years ago, the advantageous use of sensitive immunologic technics should contribute greatly to a fuller understanding of differences between tissues in biochemical terms. The question whether the malignant change involves a gain, loss, or qualitative alteration of antigenic components is relevant here and will demand further probing into almost every aspect of cell function and constitution.

Transplantation immunity need no longer be viewed as "some novel and mysterious process," but conforms in main outline with the immune state generated by infection (85, 174). A spontaneous tumor is not a foreign tissue, hence cannot evoke as measurable a response as the iso-antibodies elicited by grafts. Neoplastic antigens proper may indeed be ephemeral; yet, the reality of auto-immunization in health and disease (36, 124, 203) argues against premature abandonment of the search for them.

The literature reviewed in the present survey is representative of factual as well as theoretical developments, largely since 1942. The ramified subject matter has been organized into five chapters: (I) The immunogenetics of tumor transplantation; (II) Induced immunity and hypersusceptibility; (III) Neoplastic antigens and the question of their specificity; (IV) Immunologic approaches to therapy and protection against carcinogens; and (V) Cancer diagnosis by serologic methods. Some recent general considerations of specific antigenic entities in malignant cells (135), iso-antibodies in tumor transplantation (85), growth-inhibitory and enhancing effects of nonliving tissue fractions (200), host and extraneous factors in heterologous grafting (66), and the concept of tumor autonomy (95) should supplement the following discussion with viewpoints of sufficient variety for a balanced appraisal of current trends.

I. The Immunogenetics of Tumor Transplantation

The dependence of successful transplantation on the presence of dominant genes simultaneously in graft and host applies to both normal and neoplastic tissue. Reviews by Bittner (22) and Little (155) contain historical accounts of the evidence supporting this generally accepted principle.

If a tumor graft carries one or more dominant loci functioning in the elaboration of cellular antigens and the host has inactive or immunologically different alleles of these genes, the implant evokes antibodies against itself and usually regresses: If, on the other hand, the pertinent antigenic alleles are dominant in both tumor and recipient, no im-

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mune reaction ensues, and the host succumbs. The designation "dominant" versus "recessive" is perhaps an oversimplification (196). Both alleles could produce specific antigens, as in the case of the blood groups which, in many respects, can be considered as models for the immunogenetics of tumor transplantation.[1] Since "the specificity of antigens reflects that of genes more directly than does any other character" (220), a one-to-one relationship between antigenic end-product and gene may be inferred by further analogy with the blood groups.

Histocompatibility.—Gorer (81, 82) has postulated that the genes which govern the fate of a transplant also control iso-antigenic differences. The name "histocompatibility genes" and the symbols *H/h* (196) have been proposed for these factors, best known of which is the *H-2* locus in the A strain mouse. *H-2* and its alleles play a dual role: they determine erythrocyte antigen II (one of the four known murine blood groups) and susceptibility or resistance to certain transplantable tumors (81, 83). Sera of C57BL mice previously inoculated with A strain tumor 15091a (which regresses in the blacks) agglutinate A strain red cells. The regular coincidence of antigen II and compatibility for 15091a and other A tumors in second generation crosses between A and C57BL indicates that blood group II and histocompatibility-2 are determined by the same gene. The close linkage of *H-2* with the loci for fused tail (*Fu*), kinky tail (*Ki*), and brachyury (*T*) on the ninth chromosome of the mouse (88) has been advantageously applied in exploring the *H-2* series which now comprises five dominant alleles characteristic of several inbred strains and essential for the "take" of corresponding neoplasms (197, 202).

Inbred strains of mice and tumors which have originated in these pure lines are the best available material for immunogenetic tests. The number of factors controlling "take" can be calculated from the Mendelian percentages of tumor deaths among F_2 and backcross hybrids between susceptible and resistant stocks. This number varies with the tissues and host-strains employed and tends to diminish on continued transplantation. From the extensive literature on this subject and from his own data, Snell (196) has estimated the total number of *H*-loci in all strains of mice as at least six or seven and more probably in the neighborhood of fourteen.

[1] Most antigens in the mouse show a dosage effect, reactions being weaker in heterozygotes than in homozygotes. Occasional complete extinction in the heterozygote is conceivable. The Lewis antigens in man, which are recessive in the adult and apparently hypostatic to antigen A, are instructive in this respect (P. A. Gorer, personal communication).

The antigenic intricacies apparent from this numerical estimate and complicated still further by multiple allelism and by Snell's discovery (197) that the *H-2* locus determines several antigenic components similar to the human rhesus constellation (confirmed by unpublished serological data of Gorer), cannot easily be unraveled through the use of standard pure lines. Most of the inbred strains differ from one another in some three to eight *H*-factors; hence, the identification of individual antigens would involve stepwise elimination of antibodies by laborious technics. An ingenious way around these difficulties is forthcoming in so-called "isogenic-resistant" (IR) sublines identical with the susceptible *HH* parent stocks except for *hh*. Such IR genotypes are currently being developed on a large scale at the Jackson Memorial Laboratory (196) and at least one of them has arisen spontaneously through an *H* to *h* mutation in C57BL (27).

On the assumption that one gene elaborates no more than one antigen, graft regression and subsequent immunity in an IR subline (genotype *hh*) can be attributed to a single end-product of the gene *H*. Thus, serologic and perhaps even chemical characterization of a genetically tagged antigenic substance appears less remote. Further advantages to be gained from immunological work with a representative variety of isogenic resistant lines are: critical identification of individual *H*-factors; distinction between the antigens in different tumors; ease of correlation between blood group differences and *H*-loci; decisions regarding the relationships of dominance between *H* and *h*. The latter test requires the transplantation of tumors originating in an IR subline (genetically *hh*) into the corresponding *HH* strain, where regression should occur if the "recessive" allele produces a specific antigen (196).

Iso-antigenicity.—Through combined transplantation, hemagglutination, and differential absorption technics, Gorer (82–85) has accumulated convincing evidence for his theory that the genes responsible for iso-antigenic differences coincide with *H*-loci. Certain important iso-antigens were shown to be shared by mouse erythrocytes, normal fixed tissues and tumors, adequate care having been taken to guard against error from red cell traces in the tumor inocula. In rabbits, the antigens of skin and leukocytes are interrelated, but massive doses of skin could not elicit the formation of red cell iso-agglutinins (173). Mouse erythrocytes were chosen by Gorer as the most suitable cells for the study of iso-immune reactions, while tumors appeared preferable to normal tissue grafts on two counts: they generally require fewer

histocompatibility factors, and—possibily because of their simpler antigenic constitution—they stimulate a better antibody response.

Following the inoculation of neoplastic tissues or of blood from one pure line of mice into another (A, C57BL, BALB/c, C3H, CBA), various antibody types were determined on the basis of reactions obtained with the erythrocytes of the donor strain (83, 84). Additional agglutinins and lysins, not detectable by murine antisera, were brought to light by the enhancing properties of suitably absorbed heated and unheated human sera (86). The total picture gained from these extensive serological tests resembles the antibody categories in human rhesus iso-immunization (191). Mice form at least four kinds of antibody in response to tumor inoculation: ordinary saline agglutinins; agglutinins active only in high concentrations of mouse serum; agglutinins inactive in mouse serum but active in human serum; and antibodies which give an agglutinin pro-zone in heated human serum, but lysis with unheated human serum.

The connection between these anti-erythrocyte bodies and the defensive entities which inhibit the tumor grafts that have induced them is not fully understood and introduces the problem of partial antibodies. Ordinary agglutinins had no protective function. The neutralizing power of an antiserum for A strain leukemic cells, for instance, was not weakened by absorption of the agglutinins (83). From this it was concluded that mice also produced nonagglutinating protective antibodies. The latter could be absorbed by leukemic cells and to a much lesser extent by red cells. Despite such quantitative and functional differences, the two types of antibody appeared to be directed against the same antigen. Since the agglutinins in cold-stored sera tended to be transformed into partial antibodies of which the blocking type was the final stage, similar developmental changes of agglutinins into mature functional protective bodies may occur under natural conditions *in vivo*.

Pure genetic studies in histocompatibility are insufficient for critical evaluation of the complete antigenic structure of a neoplasm, and should be supported by serologic tests. Even after pooling the resources of available technics, one is faced with several difficulties:

1. Various iso-antigenic factors exhibit a wide range of antigenic potency (85), and certain host strains give a generally higher antibody titer than others (52) or differ in the pattern of their response (2).

2. Some grafts grow in spite of antigenic incompatibility with their hosts, while others fail to grow in favorable genotypes (85, 86).

3. *H*-loci may undergo germinal mutation (27).

4. During the course of serial transfer, tumors frequently exhibit "*en bloc*" losses of genetic requirements (155, 208), "antigenic simplification" (85), or "adaptive" changes induced by temporary residence in heterozygous host-genotypes (12, 105).

Variability of tumor cell populations.—The phenomenon of "antigenic simplification" and the much rarer instances of increased specificity (105, 157) may be approached through the general question of the relationship between antigenic instability, the cytogenetics of tumor cell populations, and selective pressure from an immunologically hostile environment.

Methods for the study of antigenic "mutability" in cancer are not limited to repeated histocompatibility testing (12, 105). Technical and theoretical guidance has come from advances in bacterial genetics. Variations in immunological reactivity of leukemic lines (157) or in their resistance to chemical inhibition are subject to statistical analysis by the microbiological "fluctuation test" (156) adapted for neoplastic cell populations by Law (148). Ascites tumors (140) also are ideally suited for such experiments, since they permit not only greater quantitative accuracy than solid growths but are relatively free from nonmalignant elements, allow segregation into clonal sublines, and lend themselves to exact correlation between some cytological and genetic characteristics (104).

Strong's (208) concept, ascribing decreased host specificity to somatic mutations in malignant cells, does not need to conflict with Gorer's (85) suggestion that an increase in one antigen might crowd out a number of others and that this masking process accounts for the functional elimination of weaker *H*-factors during the course of serial tumor passage. Gorer correctly stresses the unlikelihood of simultaneous homozygous point mutations in several pairs of *H*-loci all at once. However, the abrupt shifts, observed for instance in F_2 ratios for the dBrD tumor which transformed from a 7 via a 2 to a 1 factor requirement (209), become more amenable to interpretation if one ascribes the sudden disappearance of antigens to rearrangements of genic equilibrium arising primarly from disturbances in chromosome number rather than from intrachromosomal changes.

The cytological prerequiste for such a view is provided by the widespread heteroploidy which is apparently a universal phenomenon in all embryonic, normal adult, and malignant tissues critically analyzed for chromosome number distribution (18, 106, 143, 214). Transplantable tumors are thus not genetically homogeneous but

represent mosaics wherein mitotic aberrations provide ample opportunity not only for physical loss of histocompatibility genes, but for additive ploidy changes bringing about antigenic modification through interchromosomal disharmony.

Under continuous natural selection in compatible hosts, the cells with the most balanced chromosome sets have advantages over their aberrant competitors. This was evident from a representative series of seven mouse ascites tumors which, in spite of numerous mitotic irregularities, perpetuated their characteristic diploid or tetraploid distribution modes over more than fifty successive transplant generations (104, 106), and in one of which the average desoxyribonucleic acid value per cell remained constant for 260 successive weekly transfers (G. Klein, personal communication).

If such a tumor is inoculated into incompatible animals, survival value no longer depends on mere chromosomal balance; the genetically foreign host functions as a "concentration culture," and compatible heteroploid cells as well as $H \rightarrow h$ mutants with unimpaired capacity for division and with the minimal antigenic handicap will be selected out. Certain predominantly tetraploid tumors offer a much wider range of viable nuclear constitutions for environmental adaptation than do the diploid types. The latter were found to be very specific in their histocompatibility, while tetraploid neoplasms survived in several unrelated strains of mice (104, 107). Degree of host specificity thus resolves itself into a problem of immunological selection from among the antigenic variants in a population of malignant cells.

Some transplantable tumors, genetically *HH*, undergo permanent "adaptive" modifications during a single passage through a hybrid *Hh*-genotype (12). Although F_1 hybrids between susceptible and resistant strains generally resemble the susceptible parent in giving 100 per cent takes followed by progressive tumor growth and death of the host, the tumor is somehow altered so that upon later grafting into resistant backcross mice it will exhibit decreased or increased specificity. These induced shifts in antigenicity are usually not haphazard but may proceed from a good fit for a given number of histocompatibility genes to an equally close fit for a smaller or larger gene number (105). It is possible that the recessive loci in the heterozygous host allow the elaboration of small amounts of antibodies which interact with the surface antigens of malignant cells with antigen-precursors in the cytoplasm, or perhaps even with the corresponding causative genes in the nucleus, thereby bringing selective pressure to bear or causing actual changes in antigenic structure. Such changes could also result from exposure to "hybrid substances" like those found by Irwin (119) in his pigeon × dove crosses.

Stability of cellular antigens in different genic environments varies from case to case. The anthropoid blood group A, for instance, displays the same properties regardless of whether the causative gene operates in the protoplasm of man or ape. The ciliary antigen C of *Paramecium aurelia* (204) shows great constancy in certain clones but is so unstable in others that it can hardly be maintained. From the transformation experiments of Sonneborn and his associates it can be concluded that nuclear genes determine only which antigenic types *may* arise but do not dictate which one of a series of alternatives *does* arise. In homozygous stock of *P. aurelia*, Variety 1, only one of three immunogenetic loci comes to full expression at a given moment, penetrance depending upon the state of the cytoplasm which may be experimentally modified by slight changes in environmental temperature. In heterozygotes, on the other hand, two antigens are regularly formed by the same cell (17). Drosophila antigen 1 is the inter-action product of the wild alleles of ruby and vermilion plus additional as yet undetermined loci (74); and Irwin's (119) species hybrids produce antigenic entities different from and in addition to the parental iso-antigens. Genic interaction in a heterozygote is thus clearly capable of modifying immunologic patterns. Without violating the genetic principles of histocompatibility, upward and downward shifts in the specificity of tumors after residence in hybrid genotypes may be interpreted either as due to selection or as resulting from interference with the "cytoplasmic steady state" of antigen elaboration. If future analysis excludes these two possibilities, the less probable hypothesis of a direct mutational influence of antibodies on antigenic loci (68, 211) will bear examination.

Statistical studies of chromosome-number distribution correlated with histocompatibility genetics and clonal variance during serial transfer in susceptible and resistant genotypes should clarify the relative function of nuclear genes and cytoplasmic "cancer kappa" (136) in shaping cellular antigens. The question appears to be not so much which genic potential may gain phenotypic expression and what major antigen crowds out minor ones; but what antigenic loci are physically present, what multiples of relevant genes have arisen through heteroploidy, and what range of viable immunogenetic variants has thereby come into being.

While these generalizations are strictly limited to transplants, they overlap in principle with Boveri's (29) fundamental though unsubstantiated views concerning the chromosomal basis of tumor origin. It is no longer possible to dismiss mitotic abnormalities and the range of heteroploid variability as the pathological aftermath of established malignancy, since similar conditions also prevail in embryonic and normal adult tissues.

Weiss (217) has recently presented preliminary evidence for the self-regulation of organ growth by its own products. The mechanism involved is reminiscent of auto-immunization: protoplasmic synthesis yields "templates" for further reproduction and accessory diffusible "anti-templates" capable of inactivating the former after a critical concentration threshold has accumulated.

If one deduces from such "immunotactic" phenomena as the orderly reconstitution of minced sponges and hydroids that the spatial tissue integrity in higher organisms, too, is maintained by "antigenic" surface configurations, the physiologic isolation which characterizes cancer may have its beginnings in a kind of immunologic amnesia. Malignant invasive growth, on this view, would result from the coincidence of three chance events: viability of a chromosomal, i.e., antigenic variant tantamount to a somatic mutant, the accident of a mitotic stimulus, and the concentration of growth-regulating substances in the common humoral pool.

II. Induced Immunity and Hypersusceptibility

By judicious selection of materials according to genotype, host-tumor combinations in which neoplastic growth is prevented, retarded, or enhanced could be produced at will. The immunizing tissue need be neither malignant nor homologous with the tumor, for prior injection of embryo skin or blood inhibits malignant grafts in partially related lines; such treatment, however, fails to give protection within the inbred strain in which the tumor arose (9, 23). This implies that the mechanisms operative in transplantation immunity are not specifically antineoplastic but are influenced to a large extent by iso-antigenic relationships.

Failure to control genetic factors and thereby iso-antigenic differences has been, according to Gorer (82), the most common fundamental error in the immunological approaches to neoplasia. Experimental design can never fully conform to this criticism, for complete homozygosis is unattainable in a mammalian inbred line (101) and its tumors. The latter appear to be genetic mosaics because of wide ranges in chromosome numbers, and the hosts are subject to germinal mutation in *H*-loci. Yet, rather rigid genetic control, at least with regard to histocompatibility factors, is practicable for limited periods of time.

The few cases in which animals of adequately inbred strains were immunized against autogenous growths (5, 79, 96, 160, 161) seem to have been facilitated by mutational differentiation of either tumor or stock during long periods of serial transfer (200). Accepting this interpretation of her earlier success with induced resistance to a rat lymphosarcoma (79), Goldfeder has recently shown that x-ray attenuation of a preliminary graft of Sarcoma 180 protected *heterogeneous* Swiss mice against subsequent implants of S-180; however, irradiation of mammary tumors of A and DBA origin was without immunizing effect in the corresponding pure lines (80, and personal communication).

The prolonged brother-sister mating in the host-strains of Aptekman and Lewis (8), or the susceptibility of all nonimmunized control rats to King A fibrosarcoma and Lewis sarcoma 3, is no assurance against minor immunogenetic differences. These, then, would favor a refractory state following oncolysis by means of alcoholic tumor extracts (6) or tumor atrophy caused by strangulation (152). It is interesting that tying off the blood supply for as little as 24 hours induced immunity, whereas surgical excision of an untreated sarcoma or carcinoma after 8–13 days of growth did not bestow resistance. The degree of response to oncolytic prophylaxis could be selected for, so that the percentage of immunity (82 per cent) inducible among the offspring of successfully treated mothers was greater than that (50 per cent) in litters from nonselected parents (6, 7). A continued genetic differential between host-stocks and tumors employed is thereby indicated and was inadvertently reinforced—despite inbreeding—by selection for those genotypes which, because of their greater responsiveness to the immunizing treatment, were apt to be antigenically most distinct from the tumors.

The postulate of Gross (97) that "tumor immunity is directed specifically against the immunizing tumor, as such, and is not caused by genetic differences between the cells of the host and those of the animal in which the tumor originated" was invalidated by his own unsuccessful attempts (98) to immunize C3H/Bittner mice against three carcinomas autogenous in this strain. Intradermal inoculation of these same three tumors could, however, evoke resistance in the sufficiently distant C3H/An cousins.

Neoplasms propagated in mixed stocks elicit antibodies that are directed against them not as

malignant growths, but merely as cells derived from genetically different individuals (67). This fact detracts from investigations based on material such as the Ehrlich mouse carcinoma (53, 99, 151), the Yoshida rat sarcoma (179), and the Brown-Pearce rabbit carcinoma (56, 135). Protection against the latter tumor has been achieved not only by preliminary intracutaneous grafting of subthreshold amounts of Brown-Pearce tissue but also with embryonic skin (37); as in many similar experiments, the induced immunity is, therefore, nonspecific. Embryonic inocula genetically identical with a given tumor can immunize against it only in animals of a partially alien hereditary constitution, as shown for a mammary carcinoma in rats of the inbred August strain (67). Fetal cells which are genotypically distinct from both tumor and corresponding hosts may also function as prophylactic antigens. Wistar rats, for instance, have been rendered resistant to an indigenous lymphatic leukemia by pre-treatment with unrelated embryonic substance from Rockefeller hooded rats (210). A single intraperitoneal injection of fetal skin, viscera, or adult liver from strain STOLI (but not from strain C58) enabled C58 mice to resist an otherwise lethal challenge of C58 line I leukemia (157).

The long-range studies of MacDowell and his associates (157) on variations in leukemic lines represent the most thorough analysis of its kind on record, having dealt with several independent variables, including immunologic reactivity. Before the 674th transfer, line I leukemia was less uniformly responsive to the STOLI treatment than it has been ever since. Prior inoculation with STOLI tissue also rendered C58 mice antagonistic to four other transplantable lines of C58 leukemia (M-liv, M-kid, A, L), but was without effect against lines S, T, U and against first transfers of fourteen spontaneous cases. Although the slowly invading relatively avirulent cells taken from spontaneous C58 leukemias were not overwhelmed, while the highly virulent line I cells were, the establishment of resistance did not appear to hinge on the degree of virulence. Broadly, those lines with the capacity to induce immunity to themselves were also crossreactive with STOLI material. This acquired immunity was totally inadequate in preventing the later occurrence of spontaneous leukemia, which fact reiterates the dependence of immunization on the cumulative genetic gap resulting from long-continued serial transfer.

Passive transfer of acquired resistance (157, 188) could be accomplished for lines I and U, but not for line S (which was also nonresponsive to the STOLI treatment), by intraperitoneal injection of minced liver from an experimentally immunized mouse followed at once by a challenging lethal tumor dose. Whereas, in the case of mouse leukemia, living cells were required for the transfer of protection, Nettleship (181) has reported regression of the Murphy rat lymphosarcoma after inoculating large quantities of heterologous rabbit antiserum; small amounts were ineffective. The titer of antibody transmitted appears to have been the deciding factor in various attempts at passive immunization of susceptible animals through parabiotic union with resistant hosts (21, 77, 108). Blood exchange between parabionts, as determined from labeled erythrocytes, was 100 times greater in homogenetic than in heterogenetic pairs, which observation explains the failure to transfer immunity to heterogenetic partners and the discrepancies between several investigations of this type (21). It has even been found that natural resistance can be overcome during parabiosis of a normally refractory animal with a susceptible individual (44, 45). Whether the latter phenomenon and similar results following foster nursing (13, 15, 43, 145, 146) depend on the transfer of metabolites, cells, or virus is undecided at present.

The function of the antigen in tumor immunity induced by red cells seems to be "based upon the architectural integrity of the cell, its surface or some major fraction thereof"; for the power of strain DBA erythrocyte suspensions to elicit resistance against sarcoma DBA 49 in BALB/c mice was destroyed after laking slowly frozen cells in distilled water or after disintegration with high-pitched audible sound. Intact red cell ghosts, however, retained their antigenicity (14).

Whether dead tissue or tissue products can immunize against a tumor implant was a question much debated by the earlier reviewers (206, 219), but this problem has since been settled in the affirmative. A recent survey by Snell and his associates (200) cites eight examples of tumor inhibition and twelve instances of growth enhancement —including Casey's well documented "xyz-effect" (35)—after pretreatment with nonliving substance. The most probable explanation for the diversity of experimental results is the complex genetic basis of tissue specificity, rather than the variety of methods employed to kill and extract the immunizing tissue.

In extensive experiments carried out at the Jackson Memorial Laboratory (200, 201), mice which had received several injections of frozen-dried tumor or normal tissue were implanted with living tumor. The important variables in these tests were: the tumor used as lyophilized antigen, the antigenic dosage, the host strain, and the liv-

ing tumor graft. Results depended primarily on the particular coincidence of the genetic variables, a total of 45 different host-lyophilized tissue-tumor challenge combinations having been tested repeatedly with predictable outcome. In the C57L stock, for example, prior injection of frozen-dried leukemia C1498 (C57BL origin) gave almost complete inhibition of tumor growth. Conversely, if the tumor was A strain carcinoma 15091a and the host C57BR, prior injection made the normally refractory brown mice 100 per cent susceptible. Some lyophilized tumors, notably fibrosarcoma L946 and mammary carcinoma E 0771, retarded the challenge grafts in certain host genotypes and enhanced them in others. Comparable results have been recorded for cell fractions produced by differential centrifugation (198) and with tissue antisera (127). The difference between resistance and hypersusceptibility was sometimes merely a matter of small versus large dosage of antigenic material (128, 129).[2]

In a critical summary of this work, Snell (199) has drawn relevant parallels between Casey's data on growth enhancement of the Brown-Pearce tumor (35), Felton's (70) paradoxical results with pneumococcus polysaccharide ("immunological paralysis"), Owen's (182) observations on identity of blood groups in fraternal cattle twins, and the enhancing phenomenon in homoiotransplants of mouse tumors. The latter effect is species-specific and relatively strain-specific, stimulation being most pronounced when the normal lyophilized tissue and the tumor are derived from the same inbred strain. Neither induced resistance nor hypersusceptibility, however, have so far given any indication of tumor-specific antigens.

III. Neoplastic Antigens and the Question of their Specificity

The recognition, through serological technics, of authentic neoplastic antigens, is handicapped by the considerable array of antigenic entities known to be associated with normal cells: species- and organ-specific tissue antigens, tissue globulins, cathepsins, lipids, blood group substances and histocompatibility factors, Wassermann and Forssman antigens. Viral and other microbiological symbionts without etiologic relationship to the cancer process (149, 212) could lead to false conclusions, if accidentally infected tumors were to be used as test material. Differential absorption with homologous normal tissues would, in such cases, be an insufficient safeguard against error.

[2] Gorer (83) has confirmed the earlier results of Tyzzer showing that antibodies may sometimes stimulate growth. This is most likely to occur in the presence of a relative excess of antigen.

Another important source of nonspecific reactions is the anticomplementary effect of thromboplastin or cephalin (167). The latter still functions in fractions of μg/cc and therefore cannot easily be eliminated from antigens by simple dilution or by fat solvents. To confuse matters still further, many test sera contain "accelerator globulins" with a tenacious capacity for activating the thromboplastic quality of cephalins, even after "inactivation" for 30 minutes at 57°–65° C.

To the extent that these complicating factors have been taken into account, experiments directed at the immunological specificity of tumors may be considered as critical.

Viral antigenicity.—Exogenous viruses and virus-like agents, whose etiologic share in oncogenesis can be established by transmission experiments, might be expected to yield more easily to serologic definition than endogenous antigenic constituents of the malignant cell, because a virus should be recognizable to the host as foreign substance. The antigenic nature of the filtrable causative agents of avian sarcomas, the Shope rabbit fibroma and papilloma, and certain mouse mammary carcinomas has been the subject of intensive investigation, justifying the conclusion that the ability to elicit neutralizing and other more or less specific antibodies is indeed a characteristic of known tumor viruses.

Centrifugal separation methods developed by Claude (38, 39) have provided concentrated purified antigenic material for immunological study. The agent from chicken tumor I has been isolated in a high degree of purity and with unimpaired tumor-producing activity. Dependence of the latter on the integrity of ribonucleoprotein suggests that the nucleoprotein may be an essential part of the active principle (42). Comparing a particulate infectious fraction isolated from fowl sarcoma I by differential sedimentation, and a control fraction from 8-day-old chick embryos, Barrett (10) could not differentiate the two systems in rabbit antisera by the precipitation reaction, and agrees in this finding with Kabat and Furth (78, 121). Still further refinements in purification, followed by complement fixation tests of Rous and Fujinami tumor extracts from successive stages of centrifugation with rabbit anti-Rous Berkefeld filtrate and with immune sera against normal fowl plasma, have revealed serologic similarity between the two viruses, but antigenic differences between them and normal host tissue components (55).

Neutralization tests *in vivo* (10) allowed antigenic distinction between extracts of chicken tumor and chick embryo. Antitumor rabbit serum

neutralized tumor agent, but anti-embryo serum and normal serum did not. The inactivating properties of antitumor serum could be absorbed with tumor extract but not with embryo extract. Complement-fixing antibodies evoked by heavy materials from chicken tumor as well as similar sediments from normal chicken spleen are unrelated to neutralizing antibodies, for the latter were never formed against nonmalignant sediment (122).

When sera of fowls were tested 1 or 2 years after recovery from Rous I tumors, they still gave a high titer of neutralizing antibodies to the Rous agent. The demonstration of active antibody in such birds may be regarded, by analogy with other virus diseases, as evidence for the continued presence of neoplastic virus, although no further tumors are being produced (33). Hens that are carriers of the Rous virus lay eggs containing a considerable amount of virus-neutralizing antibody in the yolk. Since virus was not detectable in the eggs, embryos, or chicks derived from these carriers, however, it was concluded that transmission via the egg is possible but is probably not an important cause of the high incidence of neoplasms in poultry (34).

The difficulties in distinguishing between normal tissue antigens and latent symbiotic viruses are illustrated by immunological studies of certain chemically induced, nonfiltrable fowl sarcomas which show close antigenic relationships with filtrable agents (4, 72, 73), and by the cross-immunity between several avian lymphoid tumor strains (32). High-speed sediments of the nonfiltrable methylcholanthrene Sarcoma 16 (89) inoculated into rabbits evoked antibodies that neutralized filtrable leukosis agent 13 in the absence of complement and after absorption with normal chicken spleen. Rabbit antisera to normal fowl tissue lacked inactivating properties. The titer of antibodies in chickens neutralizing agent 13 showed an interesting inverse correlation with the growth behavior of Sarcoma 16. Sera of birds, in which the tumor had regressed or was growing slowly, blocked agent 13; rapid metastatic growth, however, was never associated with overlapping immunity. All attempts to demonstrate an infective agent in Sarcoma 16, including passage through ducklings, met with failure. It follows therefrom that the cells of this methylcholanthrene-induced tumor contained a specific antigen closely similar to constituents of agent 13 but unable to initiate malignant processes; or that the cross-reacting antigen was itself a carcinogenic virus which lost its infectivity when its intimate association with cellular elements was destroyed by filtering.

The experiments of Duran-Reynals and his associates on the age- and species-dependence of natural neutralizing antibodies against chicken- and duck-variants of the Rous sarcoma agent (60, 61, 139) and on the immunological implications of virus mutability (64) have not only revealed pronounced antigenic shifts coincident with the processes of adaptive change, but have strengthened the doctrine of an infectious cancer etiology by deriving several stable lines of histologically distinct neoplasms and leukoses from induced variations of a single virus (63). Resistance or susceptibility of host tissues which themselves undergo ontogenetic changes in antigenic specificity (31) and their multiform responses to an agent of fluctuating antigenic potential—ranging from epithelial growths and lymphosarcoma through multiple bone tumors to the induction of nonmalignant new bone—assumes the proportions of a basic problem: immunodifferentiation in histogenesis (20). Not only in the case of chicken sarcoma, but also in rabbit fibroma (62), the neoplastic effects of a virus depend on immunological factors for degree and type of expression.

Several tumor viruses, notably the rabbit papilloma agent (Shope), are intermittently "masked," hence, detectable only by serologic search for the specific antiviral antibody (131). The outcome of such indirect demonstrations, which have indicated that much less virus is present in the papillomas of domestic rabbits than in those of the wild cottontail hosts, may be obscured by extravasated antibody. Reduction or abolishment of antigenicity by extravasated antibody can give false negative results, and this possibility may explain the failure of some immunization attempts with extracts of cancers arising from the natural papillomas of cottontails. The partnership between such a cancer (V2 carcinoma) and the virus endured for 5 years of transplantation in domestic rabbits (132, 195); all later tests for the specific antibody in the blood were, however, negative. Had the virus merely ridden along as a passenger until it was lost? Apparently it was no longer essential for the continuing malignancy of the V2 carcinoma cells (137).

The Bittner milk agent, functioning synergistically with genetic constitution and hormones in the production of mouse mammary tumors (24), is highly antigenic under certain experimental conditions. Complement-fixing antibodies (19) and precipitins for the mammary tumor agent (118) have been reported. In rabbits or rats, it elicits inactivating antibody effective both *in vitro*

and *in vivo* (3, 93). If, as is probable, the milk factor is a virus, it should behave as an antigen not only toward foreign hosts, but also toward the susceptible species; in mice, however, neutralizing immune bodies could not be clearly demonstrated (87).

Green (91, 92) prepared a "cancer antiserum" by inoculating rabbits with centrifugates of C3H mammary tumors, cell suspensions of which were later incubated with the immune serum for several hours and then tested for carcinogenic activity in ZBC mice. Tumor growth was completely forestalled by incubation with the antiserum, while cancer cells from the saline control rapidly developed into fatal neoplasms in all the inoculated animals. Further controls injected with antiserum toward normal mammary tissue, or with ordinary rabbit serum, also developed transplant cancers, but at a much slower rate. This retardation was ascribed to a nonspecific factor, removable by absorption with normal mouse mammary tissue but not with mammary tumor. On the other hand, seemingly specific antibodies present in the "cancer antiserum" were absorbed by cancer cells but not by normal homologous tissue. Although these results imply that mouse cancer cells have acquired a degree of virus-induced specificity which distinguishes them immunologically from their normal counterparts, genetic differences between test- and control-antigens (C3H versus ZBC) detract somewhat from the finality of such a conclusion.

The importance of strain differences is evident from the reactivity or nonreactivity of three transplantable mammary tumors (15091A, dbrB, and L916) to rabbit antisera, depending entirely on the C3H or DBA source of the antigen (147). A definite inhibiting effect followed incubation with antisera against lactating DBA mammary glands, regardless of whether or not the glands contained the agent. No inhibition was observed for hyperimmune sera against spontaneous mammary adenocarcinoma when the donor strain was C3H.

Among the most critically designed experiments in this field are those of Law and Malmgren (150), who selected the tissues used as sources of antigens—mammary tumor and normal lactating gland—so that potential antigenic differences could not arise either from genetic constitution or from malignancy per se. C3H tumors containing the milk factor were compared serologically with agent-free C3Hb tumors, and the same two strains furnished normal lactating tissue with or without the agent. Under these stringent prerequisities, no consistent differences were found in the neutralizing titer of antisera, measured as mean survival time and as percentage of progressively growing tumors. Therefore, neither anticancer nor antiviral immune bodies need be specifically involved in the growth inhibition of mouse mammary tumors by cytotoxic antisera.

The obstacles hampering unequivocal antigenic typing of neoplastic viruses have their counterparts in other branches of virology. Some pathogenic viruses do not give complement fixation in mixtures with their specific antisera or cannot be extracted from tissues diseased by them either in sufficient amount or in suitable form for *in vitro* serological demonstration. Highly purified preparations of the PR8 and Lee influenza viruses obtained from infectious allantoic fluid after egg culture contained an antigen characteristic of normal allantoic fluid, and highly purified mouse lung PR8 virus also shared an antigen with normal mouse lung (142). One therefore should not expect that, even if specific antigenic tumor components are viruses, they may be entirely dissociated from normal protoplasmic constituents.

During the events which lead from precancerous metaplasia to malignancy, or during the course of subsequent transplantation, cells may acquire constituents not found in their benign prototypes. The V2 rabbit carcinoma originated in a Shope papilloma; yet it has appropriated a specific antigen absent from normal tissues and serologically unrelated to the papilloma virus which, it should be recalled, persisted in V2 through at least 5 years of grafting (132). Specific antibody for the virus failed to react with extracts of the V2 carcinoma, and the antibody for the abnormal constituent of V2 was nonreactive with extracts of benign rabbit papillomas (75, 76, 135).

"Distinctive" constituents of tumor cells.—The most exhaustive studies of an antigenically distinctive, sedimentable substance, regularly associated with a mammalian tumor, are those of Kidd, Friedewald, and MacKenzie on the Brown-Pearce rabbit carcinoma (130, 133–135, 138, 162). Saline extracts of this transplantable tumor yielded a serologically defined component detectable neither in normal rabbit tissues (kidney, liver, spleen, bone marrow, pus) nor in the V2 carcinoma and two sarcomas. Antibody against the distinctive constituent was found in the sera of rabbits implanted with the Brown-Pearce tumor; this antibody fixed complement in mixtures with Brown-Pearce extract down to an antigen dilution of 1:1280, while control preparations failed to do so in all dilutions above 1:10.

Analysis of the purified antigen by ultra-filtration, ultra-centrifugation, lipid extraction and

proteolytic digestion has revealed superficial similarities with some of the physical and chemical properties of the viruses: large particle size and weight, lability to heat and to pH changes, protein composition and concurrence with the microsome fraction of the cytoplasm. Although similar characteristics were shared by particulate sediments of many normal tissues (40), the Brown-Pearce protoplasmic entity was readily distinguishable from control materials by serological means (162). The noninfectiousness of extracts containing the Brown-Pearce antigen in high titer and the inability of host cells to "protect" the tumor substance against attack by its specific antibody (whereas neoplastic cells normally provide such amnesty for viruses) argue against the virus nature of the distinctive constituent. Kidd (135) prefers to view it as an autocatalytic cytoplasmic determinant of proliferative activity. This interpretation hinges largely, but somewhat inconsequentially, on the apparent antiblastic effect of the corresponding antibody exerted against Brown-Pearce cells during incubation *in vitro* and also potent *in vivo*. Rabbits previously injected with cell-free extracts containing the serologically active material resisted tumor grafts if their sera contained the specific antibody. If, on the other hand, they had failed to develop the antibody they were regularly susceptible. Experimentally induced immunity had nothing in common with the unknown factors causing spontaneous regressions. The latter occurred more often in the absence of the specific antibody than in its presence, while sera of rabbits which had spontaneously recovered from the tumor did not inhibit the malignant cells *in vitro* unless they contained the specific antibody.

It has been emphasized (134) that "the specific Brown-Pearce antibody is by definition not an iso-antibody," since it always reacted with homologous antigen and regularly failed to react with extracts of a wide variety of normal and neoplastic control tissues, whether procured from normal rabbits, from hosts with metastasizing tumors, or from animals in which the growth had regressed. Nor were the erythrocytes of rabbits in any of these three categories agglutinated by the Brown-Pearce antibody. More subtle iso-antigenic factors than might be detectable by agglutination could, however, be operative in this system. The Brown-Pearce carcinoma originally arose in a hybrid animal and was transplanted in mixed stock for over 20 years. After such a lapse of time, a considerable genetic differential can accumulate through mutational changes in both tumor and hosts. Hence, it is not surprising that complement fixation by the specific antibody is predictable only for a restricted number of hybrid genotypes—the most reliable being Rockefeller "blue-cross" and "chocolate-Dutch"—and that Kidd's serologic results cannot be repeated critically with random-bred market rabbits (120).

The experiments of Dulaney and her collaborators (58, 59) on the cytotoxic activity of antisera to normal and malignant spleen cell components gave highly significant quantitative differences not as clearly demonstrable by complement fixation procedures. In this work, Hogeboom's (114) sucrose modification of Claude's (41) centrifugal fractionation procedure for the cytoplasm of mammalian liver cells was advantageously followed. The antigenicity of normal and malignant nuclei, mitochondria, and microsomes was compared. Cytotoxic effects were measured by the survival of mice inoculated with normally lethal doses of 100,000 leukemic cells which had previously been exposed to rabbit-antisera against the various cellular components. Antisera to the nuclei or cytoplasmic particles of leukemic spleen were far more damaging for leukemic cells than were the normal spleen controls. The leukemic microsome fractions were almost specifically antigenic, in that antisera against them inactivated 90 per cent of the lethal inocula, whereas normal microsomes evoked no protective antibodies.

In a recent serologic (complement fixation) analysis of microsomes from mouse hepatoma and normal liver, quantitative but no tumor-specific differences were observed (166). Microsomes from mammary tumor and hepatoma were, however, qualitatively distinguishable, presumably because of organ-specific antigenicity. If histocompatibility factors are at all measurable by complement fixation, they probably do not concur with microsomes, for strain-specific antigens were absent from the microsomal fractions of various tissues of BALB/c, C57BL, and C3H mice.

The immunochemistry of mouse tissue components derived from adult and embryonic organs and from eight transplantable tumors has shown characteristic patterns which, according to Maculla (163, 164), permit the following generalizations: the intracellular components of certain adult tissues, e.g., liver and spleen, resembled their ontogenetic counterparts serologically, whereas adult spleen and kidney differed from the corresponding embryo organs. Antigenic relationships between malignant and embryonic tissues were very broad in some instances, highly selective in others. Six histologically distinct neoplasms possessed components in common with fetal liver, yet did not overlap with adult liver. All tumor antisera re-

acted with adult lung and spleen but not with embryonic spleen. The nucleoprotein fractions of mouse tumors were immunologically distinguishable from the nucleoproteins of normal mouse organs, as tested by a rigidly standardized complement fixation procedure. In these experiments antibody titers were enhanced by the synergistic influence of Staphylococcus toxin administered simultaneously with the tissue antigens, a technic which should be more widely employed in boosting measurable responses to weakly reactive constituents of malignant cells.

Comparative precipitin, complement-fixation, and anaphylactic tests (171) showed the cathepsins of healthy rat liver and of a transplantable hepatoma to be different proteins with some common groups, and to overlap partially with the intracellular proteases in rat kidney, spleen, and Jensen sarcoma. Much more normal liver cathepsin than hepatoma cathepsin was required to produce shock in guinea pigs sensitized with hepatoma protease.

In addition to the largely quantitative antigenic differences between malignant and nonmalignant cell particulates, nucleoproteins, and proteolytic enzymes, there is some scattered evidence for the existence of relatively specific lipoidal tumor antigens (184). Hoyle's (116) alcoholic extracts of Sarcoma 37, tar Carcinoma 2146, and the Mal sarcoma gave complement fixation with the sera of mice bearing these growths but not with control sera. No comparable lipoid antigen was found in normal mouse tissues. A specifically reacting component, common to transplantable mouse carcinoma 15091a and several spontaneous and induced tumors but absent from normal mouse liver, kidney, brain, and sera, was soluble in ether and alcohol (30). That lipids and lipoproteins may indeed be basically concerned in the antigenic individuality of neoplasms is further borne out by the successful immunization of rats against transplantable sarcomas of closely corresponding genotype, following a course of injections with alcoholic tumor extract (5, 6, 8, 153). Such extracts do not always interfere with the growth of treated tumors, but can produce great enhancement (69).

Very little is known concerning the antigenic behavior of autochthonous growths, both induced and spontaneous, within the systems in which they arise. There is some indication of defensive reactivity specifically directed against spontaneous mammary carcinoma in mice; and, although this weak immunity could not suppress the primary growth, it appeared to interfere with metastases, for autologous transplantation of bits of primary tumor to other sites in the same animal often failed (100). This was not an athreptic phenomenon due to nutritional depletion of the host, since surgical removal of the primary growth did not improve viability of secondary implants and since takes of homoiotransplants (25) and even of heterologous neoplasms such as the Rous sarcoma (94, 95) were more easily obtained in mice already supporting spontaneous tumors of their own than in normal controls. Under such conditions, the increased susceptibility to genetically foreign implants was perhaps expressive of the general decline in antibody production found associated not only with well established malignancy (183, 218) but occurring soon after the application of certain carcinogens (113, 165).

In retrospect, the serological studies of tumors, though extensive, were seldom controlled in a way which (except in the case of some viruses) would allow unequivocal isolation of specific neoplastic antigens. Where claims for specificity have been made, the genetic gap between tumor and host has generally been objectionable. The evidence for quantitative if not qualitative differences in the antigenic components of normal and malignant tissues is, however, convincing. Not only are the immune bodies described (complement-fixing and neutralizing antibodies, precipitins, lysins, agglutinins, and antiblastins) of the same types as those familiar to microbiologists, but the similarity between tumor immunity and resistance against parasites is further emphasized by the protective functions which cellular elements of the reticuloendothelial and lymphatic systems perform in both neoplasia and infectious disease (177, 178, 192, 207). The lymphocytic theory of antibody formation, as revised by Ehrich and Harris (65), is in keeping with observations on the coincidence of tumor decline and lymphocytic activity, as well as with the finding that there is a reversible exchange of antibody between normal and malignant lymphocytes and that the latter may even exceed the former in their demonstrated capacity for antibody production (57).

IV. Immunologic Approaches to Therapy and Protection against Carcinogens

Although effective immunization against transplantable tumors—mouse leukemia, for instance—does not interfere with the strain-characteristic incidence of spontaneous neoplasia of the same histologic variety as the immunizing graft (158), and although passive transfer of experimental immunity by means of cell-free sera (159) or therapeutic trials with antisera "specific" to various tumors and homologous normal tissues (28) have generally met with failure, a hopeful attitude

relative to direct serologic therapy persists to this day in some quarters.

Domagk (56), despite the negative outcome of his own attempts to immunize inbred high-cancer lines of mice against spontaneous mammary adenocarcinoma, proposes a clinical evaluation of undenatured cold extracts made from human neoplasms. Having demonstrated the seeming harmlessness of such preparations by injecting himself "many times" subcutaneously 10 years earlier, he recommends that surgical clinics should have available blood donors willing to be inoculated with antigen from malignant operative specimens. Antisera from such volunteers might protect the corresponding patients post-operatively against metastases. This suggestion has been criticized on protein-chemical grounds (16) and is also open to ethical objections in view of the many unknowns regarding latent "Krebserreger."

The apparent role of reticulo-endothelial elements in defensive phenomena around and within foci of cancer tissue has been the guiding impetus in the work of Bogomolets and his school (26) with antireticular cytotoxic serum (ACS), small doses of which may stimulate the cellular protective reactions. ACS was prepared by inoculating horses with spleen and bone marrow from healthy persons who had met sudden accidental death. Its use is said to have brought about the disappearance of metastases and to have considerably diminished recurrences after operations for gastric and lung cancer. Tests with ACS in other laboratories showed inhibition or enhanced malignancy (depending on dosage) of the Brown-Pearce rabbit carcinoma (176) and a reduced incidence of spontaneous mouse mammary adenocarcinoma (111), but failed to affect the growth of Carcinoma 2426 and Fibroma 2011 in rats, or Sarcoma 180 and mammary tumors in mice (109, 110). Reticulo-endothelial immune serum (REIS), a preparation basically similar to ACS, inhibited Walker rat sarcoma 319 *in vitro*, provided homologous rat spleen had served as antigen (187). Sarcoma cells were not inhibited by intimate contact with splenic fragments growing in the same flasks; but in these two-membered cultures REIS injured sarcoma at concentrations lower than those needed for damaging effects in the absence of spleen.

Treatment with bacterial toxins (180) and with Shear's *Serratia marcescens* polysaccharides (194) has several important immunologic implications and complications. The polysaccharide branch of chemotherapy is traceable to observations of local tissue reactivity, i.e., the Schwartzman phenomenon, in a transplantable liposarcoma of the guinea pig experimentally sensitized with bacterial filtrate. The tumor responded to intravenous challenge by hemorrhage and necrosis (as have many mouse tumors and some human neoplasms since then), while normal host tissues remained relatively unaffected. Since the bacterial toxin elicited equally extensive hemorrhagic disintegration in the nonsensitized control tumors, prior accidental sensitization by a cross-reacting microorganism or virus was postulated (90). The potential propagation of a sensitized cytoplasmic state over many cell generations and serial transfers from host to host even in the prolonged absence of the offending allergen has not been ruled out in subsequent work with purified polysaccharides because of the impracticality of entirely germ-free technics.

A large investment of co-operative effort between laboratories and clinics has gone into screening a range of antigenically distinct polysaccharides (so far only with sporadic clinical results), and into attempts to detoxify them and to separate their potent antigenicity from their oncolytic property by chemical modification (47). It is now possible to protect mice passively by anti-polysaccharide sera against the usual toxic syndrome without decline in the tumor-destroying capacity of the antigen (49).

Both *Serratia marcescens* culture filtrate and Shear's polysaccharide are capable of eliciting the phenomenon of local skin reactivity. Schwartzman (193) found no measurable alteration in antigenic specificity (immunizing value, precipitation, and neutralization reaction) consequent to tryptic digestion and other purifying methods employed by Shear. Hence, the principles producing the Schwartzman phenomenon are akin to or identical with the factors inducing hemorrhage and regressions in certain mouse tumors.

The hemorrhagic response of experimental tumors is, however, by no means specific to bacterial products, since it has also been evoked through histamine or through anaphylaxis in mice sensitized to normal filtered horse serum (11).

The demonstration of antibodies against body secretions, organs, components of normal tissues, and distinctive constituents of some tumors (135) has suggested the potential usefulness of immune bodies for selective localization of metals (172), dyes, and radioactive tracers in malignant lesions. Assuming, for the purpose of discussion, that isoantigenic inequalities exist between hepatoma and normal liver, for instance, the difference is certain to be quite subtle. Nevertheless, under the synergistic influence of adjuvants, such as staphylococcus toxin, titers against weak tissue antigens

have been stepped up in the donors of antiserum (163), and after differential absorption a relatively specific globulin fraction could conceivably be isolated and isotopically labeled.

First steps in this field have been taken by Pressman and his associates (189, 190), who have obtained zones of localization of radio-iodinated antibody in mouse and rat kidney. By comparing radioautographs and counts of kidney slices after treatment of rats with (*a*) I^{131} antirat-kidney serum globulin, (*b*) I^{131} anti-ovalbumin, and (*c*) combined I^{131} anti-ovalbumin and nonradioactive iodinated antirat-kidney globulin, it was shown that localized radioactivity in the kidney is due to a specific substance, presumably an antibody. A cytotoxic effect of antiserum causing the glomeruli to pick up nonspecific iodinated protein was definitely ruled out.

No significant differences in the distribution pattern in mice were noted between I^{131} normal rabbit globulin and I^{131} gamma globulin from a rabbit immunized with mouse testicular cells, when the radioactivity in the various organs was assayed 21 hours after inoculation (112). From these negative results it was tentatively concluded that the physiological character of the glomeruli is perhaps indispensable to successful localization of antibody and that specific uptake in organs other than the kidney or in tumors "might fail even where striking immunological specificity existed in the bulk of the structure."

Landsteiner's (144) classical experiments achieved antigenic specificity of otherwise inactive molecules of known structure by their conjugation with proteins. The problem of an immunological defense against carcinogenic processes through haptenic activity of carcinogen-protein conjugates offers challenging possibilities for immunochemical synthesis and co-ordinated prophylactic experimentation. Creech and others (46, 50) have coupled various serum albumins with isocyanates of a representative range of polynuclear aromatic hydrocarbons. Injection of the conjugates into rabbits elicited antibody which was precipitable by conjugates of the homologous hydrocarbon with a heterologous protein. These serological reactions were entirely dependent on prosthetic group activity, hence strengthened the feasibility of protection against carcinogenesis by chemo-antigens. A series of 35 mice injected with 1,2,5,6-dibenzanthryl-9-carbamido casein has given tentative indication of enhanced resistance toward tumor induction by subsequently administered 1,2,5,6-dibenzanthracene. An overwhelming challenging dose of 9,10-dimethyl-1, 2-benzanthracene may have been responsible for the failure of homologous immunization in a preliminary trial with rabbits. More critical current tests with C57BL mice (inbred for a low spontaneous tumor incidence but very responsive to treatment with carcinogenic compounds) should evaluate the hypothetical protective efficacy of conjugates recently synthesized from proteins and certain systemic carcinogens (48, 51).

V. Cancer Diagnosis by Serologic Methods

Cancer appears to differ biochemically from normal tissues in a quantitative rather than a qualitative sense, which bespeaks synthesis not of specifically abnormal substances but of normal molecules at the wrong time and in wrong places and amounts (215). So far, the search for minute increments of cryptic tumor proteins and other unknown metabolites amidst the massive output of normal cells during early stages of neoplasia has defied electrophoretic, ultracentrifugal and solubility methods (117). Even during advanced stages of the disease, electrophoretic study of plasma proteins (175, 185, 186) and nitrogen and lipid analysis of plasma fractions (54) have revealed no characteristic deviations from the patterns of other ailments (except in multiple myeloma). Generally, the albumin concentration decreases and the alpha-globulins and fibrinogen increase, while the shift in beta- and gamma-globulins is not appreciable before the onset of cachexia.

It is not surprising, therefore, that diagnosis based on immunologic principles rather than on much less sensitive chemical methods has continued to challenge experimentation. The optimism behind these attempts is founded on the immunologic specificity of certain normal tissues, on demonstrations of more or less "specific" antibodies evoked by transplantable tumors, on the antigenicity of some neoplastic viruses and of carcinogen-protein conjugates, and on the existence in human serum of natural auto-antibodies formed against antigenic lipids which are liberated in tissue wear and tear (124). Arguments in favor of sero-diagnosis are strengthened by the continued failure of physico-chemical analysis in differentiating even between such genetically and serologically distinct entities as the human blood group substances A and B.

In spite of the plausibility of this reasoning there is as yet a great paucity of data on the antigenicity of malignant growths in man. Mann and Welker (168, 169, 170) have studied precipitin reactions between autolysates of various human neoplasms and homologous antisera formed in rabbits against intramuscular deposits of carefully

dissected and washed mince adsorbed on aluminum cream. About half the sera were free of antibodies to nonspecific serum-proteins at the end of 6 months, at which time they were titrated against autolysates of the respective malignant and control tissues. While a few of the antisera responded exclusively to homologous autolysate, the majority of the precipitins was disappointingly nonspecific.

Abderhalden's "defensive proteases" (1) in blood and urine allegedly cause a specific breakdown of those proteins in response to which they are built by the organism. The originator of the protease tests which are intermediate between immunologic and enzymatic procedures sees in the "Abwehrsproteinasereaktion without doubt the best means yet known for the diagnosis of cancer"; but evaluations by others (213, 216) have given many false negatives.

A recent review of cancer diagnosis by Homburger (115) includes brief discussions of urinary "antigen" against Aron's adrenotropin (71), cytotoxins, hemolysins, cytolysins, antibody fixation by guinea pig serum, complement fixation, skin tests with "cancer fatty acids," and diagnostic growth inhibition of tissue cultures by cancer sera (141). After a survey of these experiments, one is left with the conviction that no test of proved value in primary diagnosis has been developed.

Accessory, differential diagnosis may gain support from serological methods in special instances. Thus, Southam, Goldsmith, and Burchenal (205) have strengthened the reliability of the Forssman reaction in distinguishing between infectious mononucleosis and occasional confusing cases of acute leukemia with relatively high heterophile antibody titers. The routine sheep erythrocyte agglutination test was inadequate in these instances; however, through absorption with guinea pig kidney, antibody of the true Forssman type could be eliminated and differentiation between mononucleosis and leukemia became certain.

The potential diagnostic value of steroid antigenicity has recently begun to attract attention. An unsaponifiable antigenic fraction obtained from human cancerous livers was added to 6,400 sera, giving 2 per cent positive reactions in the general population and 90 per cent positive results in cases of histologically proved malignancy (102). Certain abnormalities of serum albumin often associated with tumor growth, such as decreased coagulability, solubility, and combining power for fatty acids, seem to depend on the presence in cancer sera of "characteristic abnormal lipids" which unite with the albumin (103). Kahn (123) tested an as yet limited number of sera from cancer patients for his "universal reaction" with two lipid antigens, one of which was conservative while the other was highly sensitive. The tumor sera resembled specimens from certain infectious diseases, in that they showed considerably more precipitation with the sensitive than with the conservative antigen, while the majority of normal samples produced the same degree of precipitation with either of the two antigens. In a more recent investigation (154), patterns for lymphosarcoma and adenocarcinoma demonstrable by this technic have been attributed to the increased wear and tear of tissue in general rather than to the release of any lipid specific for cancer. Levels of the "universal reaction" in irradiated patients (125, 126) may, however, be of definite aid to the clinician in interpreting the results of radiation therapy. These observations of immunity to lipids or lipo-proteins should stimulate further study of lipid serologic reactivity in the various forms of neoplasia.

CONCLUSIONS

In the foregoing five sections the questionable specificity of tumor antigens has furnished the central theme. A detailed discussion of immunogenetic principles, understanding of which is essential for the orientation of future experimental design, has formed the basis for evaluation of the virologic, chemical, and clinical aspects of tumor immunity. The ambiguities in the data now available are due in large measure to the use of conventional materials: long-propagated neoplasms and unsuitable host stocks. Furthermore, a priori notions regarding distinctive constituents of cancer cells reinforced by the isolation of such purported entities in genetically unfit systems, have impeded unbiased analysis. The attack on this Protean problem has much to benefit from more unequivocal immunologic methods (167), and such promising biologic tools as Klein's spectrum of ascites tumors (140) and Snell's isogenic resistant strains and histocompatibility markers (196). The minimal genetic and serologic requirements for controlled investigation should be evident from a number of model experiments included in this survey. Eventual demonstration of specific neoplastic antigens appears dubious, since the critical results to date have been largely negative. The continuing search should, however, be rewarding to the extent that pragmatic aims are guided by a primary concern with the general problem of tissue specificity.

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Localization of Sulfapyrazine in Cancer Tissue upon Glucose Injection*

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The possibility of precipitating in cancer tissue a compound administered at sites distant from the tumor was suggested by the facts that (*a*) many compounds are less soluble at acid than at alkaline pH (9, 12) and (*b*) cancer tissue is possibly unique in becoming quite acid, often below pH 6.4 as measured by glass electrode, after glucose injection (1, 10, 18, 19). The compound used, sulfapyrazine,[1] was chosen because of its low toxicity (14), its low aqueous solubility (6), its seven- to eightfold greater solubility at pH 7.4 than at pH 6.4 (8, 13), its capability of being readily measured in tissues, and because sulfonamides had been used in earlier work here (3). Experimental conditions are described in which sulfapyrazine localized in tumor implants of rats with Walker tumor 256.

EXPERIMENTAL

Solubility of sulfapyrazine.—Twenty-four 105-mg. aliquots of sulfapyrazine were placed in 90-ml. aliquots of NaH_2PO_4-H_3PO_4 buffer (0.01 and 0.1 M) and shaken 3 days at 37°–38° C. Filtered aliquots were analyzed periodically for sulfapyrazine (4), and pH values were measured with a glass electrode at 37°–38° by comparison with Bureau of Standards 0.05 M potassium acid phthalate (pH 4.03). The addition of 10 mg. of sodium sulfapyrazine monohydrate to each flask, followed by shaking overnight, gave concordant results at slightly higher pH values. The 72 measured solubilities (from pH 5.8 to 7.5) gave by least squares (deviations in solubility minimized):

$$\text{mg sulfapyrazine/100 ml} = 0.93 + (0.22 \times 10^{-5} \times 10^{pH})$$

with a standard error of estimate of ±2.0 mg sulfapyrazine/100 ml. Solutions supersaturated by heat or by excess sodium sulfapyrazine remained so for weeks, particularly if above pH 7, though solid sodium sulfapyrazine monohydrate and solid sulfapyrazine were both present. Sulfapyrazine was thus about 8 times as soluble at pH 7.4 as at pH 6.4. A 7.3-fold difference was anticipated from theory (9, 12) and the reported pKa of 6.04 (2). At pH values between 7.2 and 7.7, 10–20 per cent lower solubilities than those reported here have been found, with more carefully purified sulfapyrazine than that we used.[2]

Animals.—Of 423 large Sprague-Dawley rats, 74 died before their scheduled killing times and were discarded. Of the remaining 349 rats, 257 were tumor-bearing, 75 were used in toxicity tests, and 17 were used in tests of analytical methods. They were fed Purina Fox Checkers and tap water ad libitum. Walker tumor 256[3] was implanted, subcutaneously to simplify dissection. Each of the first 205 tumor-bearing rats had a single implant; in the last 52 rats two separate implants were made. Implants were in the scapular region as far as practical from the site of sulfapyrazine injection—the hind leg. At sacrifice, the 295 tumors had a median weight of 1.28 per cent of the body weight; none more than 13 per cent.

Sulfapyrazine was injected subcutaneously as a hot (60°–70° C.) aqueous solution of sodium sulfapyrazine containing 50 gm of sulfapyrazine/100 ml. Glucose was injected intraperitoneally. At first, when only a single injection of glucose was given, it consisted of 600 mg/100 gm of body weight in 20 per cent aqueous solution; this followed an earlier practice (1, 10, 18, 19). Later, when two injections per day were given, each consisted of 500 mg of glucose/100 gm of body weight in 50 per cent aqueous solution.

Animals were killed by taking heart blood under ether anesthesia. The tissues were at once removed, blotted, and weighted to within ±2 per cent. With tissues weighing less

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[1] The sulfapyrazine, sodium sulfapyrazine monohydrate, and acetylsulfapyrazine essential to this work were generously supplied by Dr. R. C. Ellingson of Mead Johnson & Co.

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[2] Personal communication from Dr. R. C. Ellingson.

[3] Walker tumor 256 was kindly provided by Dr. Clarence Lushbaugh and, later, by Drs. John B. Storer and John Green of the Department of Pathology, University of Chicago.

than 50 mg., this error was nearer ±10 per cent of true fresh weight. In the last 52 rats both necrotic and non-necrotic portions were taken from 57 of the 90 tumors—those from which samples of over 50 mg. could be readily obtained. The glandular portions of the stomachs were dissected free of the forestomachs and cleaned by blotting with gauze. Urine samples were drawn from the bladder.

To measure toxicity, 51 rats were injected with 50 per cent sulfapyrazine as a solution of the sodium salt at 60°–70° C.; 17 given 400–1,000 mg sulfapyrazine/100 gm of body weight died within 7 hours; 23 given 200 mg. lived 9–61 hours (median, 24 hours); eleven given 105–175 mg. lived 46–85 hours. Twenty-four others received a 40 per cent solution at 40°–50° C.[4] in doses of 75, 90, 100, 105, 110, and 125 mg/100 gm of body weight, and survived indefinitely. (Three or more rats received each dose.)

Analytical methods.—Analytical methods for the determination of sulfapyrazine were modifications of the procedure of Bratton and Marshall (4). Tissues from the first 40 tumor-bearing rats were promptly ground with sand, mixed with water, and treated with trichloroacetic acid. Those from the next 165 rats were diluted 1:20 with 1 per cent sodium hydroxide and digested at 35°–40° C., often for several days, and the digests were diluted 1:5 with 15 per cent trichloroacetic acid. Blood and serum were diluted 1:20 with 3.16 per cent trichloroacetic acid without preliminary digestion. Finally, with the last 52 rats, blood and serum were diluted 1:40 with water (which gave better recovery than alkali) and analyzed the day they were obtained, while tissues were diluted 1:40 with 1 per cent sodium hydroxide and digested, then diluted 1:5 with a solution of 3 gm of *p*-toluenesulfonic acid monohydrate[5]/100 ml; and all analyses were completed within 24 hours. Filtrates were diluted, generally 1:5, with approximately 0.13 N hydrochloric acid, diazotized, and coupled. Color density was measured within a half hour with an Evelyn colorimeter. A 5 per cent greater color density was obtained from sulfapyrazine solutions if *p*-toluenesulfonic acid and sodium ion were replaced by water in the above procedure.

[4] Use of a cooler solution was kindly suggested by Dr. J. W. Wilson of Brown University.

[5] Most lots of *p*-toluenesulfonic acid tested gave color or turbidity in blank determinations and had to be purified or "decolorized." Sometimes this could be accomplished by boiling the 3 per cent solution several minutes with 0.1 gm of sodium nitrite and 0.3 ml of hydrochloric acid/100 ml. In other instances purification was effected by recrystallization from ethyl acetate, with the use of charcoal and a little sodium nitrite. When this did not suffice, neither did simple recrystallization from a mixture of sulfuric and hydrochloric acids (5). It was necessary to prepare the sodium salt, precipitate it with concentrated alkali, filter, and dissolve it in a minimum quantity of water, then filter off an insoluble material. To the filtrate was added, with cooling, an equal volume of sulfuric acid and about half as much hydrochloric acid (enough to give maximum precipitation).

Lability to heat in various acid and alkaline solutions was measured for both sulfapyrazine and acetylsulfapyrazine. Analytical recoveries of both compounds were found to be better after heating in alkaline than in acid solutions. Trichloroacetic acid and, to a lesser degree, *p*-toluenesulfonic acid interfered with recoveries. Similar interference of trichloroacetic acid has been encountered with pyrimidine derivatives of sulfanilamide (20). In part because of these difficulties, further work on acetylsulfapyrazine was not undertaken, except for a few tests that suggested that less than 10 per cent of the sulfapyrazine present in tissue was in acetylated form.

Analytical recovery of sulfapyrazine from tissues to which it had been added *in vitro* varied from 80 to 110 per cent. Average recoveries (four rats), with approximately 200 mg of sulfapyrazine added/100 gm of fresh tissue, alkaline digestion of tissues, and precipitation with trichloroacetic acid were: hide, 103 per cent; serum, 98 per cent; whole blood, 97; stomach, 97; testes, 96; leg muscle, 96; tumor, 95; spleen, 94; lungs, 89; kidneys, 89; liver, 87; and heart, 84 per cent. Average recoveries (four rats) with 50 mg of sulfapyrazine added/100 gm of tissue and with *p*-toluenesulfonic acid as precipitant were: hide, 107 per cent; serum, 102; non-necrotic tumor, 100; testes, 99; spleen, 98; necrotic tumor, 97; stomach, 97; leg muscle, 97; kidneys, 95; lungs, 91; liver, 91; heart, 90; whole blood, 88 per cent. By the use of this analytical procedure on tissues from three of these same rats, without addition of the sulfapyrazine, the average intensity of color obtained was equivalent to the following number of mg of apparent sulfapyrazine/100 gm of tissue (tissues in same order, respectively, as in the preceding sentence): 2.0, 0.9, 0.4, 0.6, 0.8, 0.7, 0.5, 0.4, 0.7, 0.7, 0.7, 1.5, and 0.6. The tissue concentrations reported in this paper have not been corrected for any of the losses or gains described above. Duplicate aliquots agreed well: the second aliquot lay within ±2 per cent of the first aliquot in two-thirds of the analyses of the last 52 rats.

The difficulty of measuring high serum sulfapyrazine concentrations resulted in values lower than the true serum concentrations in several instances among the first 205 rats, as a comparison to muscle concentrations indicates. The concentration of sulfapyrazine remained constant in stored serum and in stored oxalated blood for several days. But if whole blood, clotted or oxalated was kept stoppered overnight, either at room temperature or in the refrigerator, the sulfapyrazine concentration in its serum or plasma occasionally decreased by 10–40 per cent. This occurred whether sulfapyrazine was present *in vivo* or was added to drawn blood. It occurred more often with higher serum concentrations. It was to avoid this difficulty that serum and blood from the last 52 rats were diluted promptly and analyzed the day they were taken.

As another test of analytical methods and for general information on tissue distribution of sulfapyrazine, total analytical recoveries of sulfapyrazine from four rats were measured after injection of sodium sulfapyrazine (Table 1*). Recoveries of 87–110 per cent were obtained, 1–2 per cent appeared in the excreta in a day's time, and high concentrations were found in the stomach, the kidneys, and at the subcutaneous injection site. A low serum concentration (presumed to be spurious) was found for one rat (D).

RESULTS

In the first experiment, 205 rats were each injected once with sulfapyrazine (Table 2). Half also received a single glucose injection. Concentrations of sulfa in the tumors exceeded those in the serums in 55 rats—26 of those given glucose and 29 not given glucose. Rats given glucose had lower con-

TABLE 1

Recoveries of Sulfapyrazine from Four Rats Injected with Sodium Sulfapyrazine

Rats	A	B	C	D	A	B	C	D
Wt. of body parts	192	257	323	382				
Mg. sulfa injected	385	398	615	1,016				
Tissue	Per cent inj. sulfa found				Mg sulfa/100 gm fresh tissue			
Blood (withdrawn portion)	1.14	1.43	2.4	1.19	168	239	167	126
Serum					260	317	221	126
Chest fluid	2.6	4.9	1.38	0.56	191	315	141	168
Stomach, forestomach and contents	1.11	0.65	2.98	0.50	64	66	795	115
Small intestine and contents	4.6	3.7	3.83	2.55	179	156	78	302
Large intestine and contents	1.56	1.56	0.41	0.45	188	155	155	238
Caecum and contents	1.45	1.38	0.98	0.85	145	100	23	266
Pancreas	0.62	0.63	0.26	0.40	200	207	107	223
Spleen	0.52	0.35	0.06	0.30	181	149	108	191
Liver	6.2	5.0	2.3	2.6	190	201	171	264
Kidneys	1.16	2.5	1.20	2.20	221	440	205	483
Thymus		0.10	0.04	0.04		169	181	112
Lungs	1.06	1.01	0.42	0.72	271	196	156	304
Heart	0.44	0.53	0.28	0.47	257	221	160	368
Testes	1.12	1.11	0.42	0.43	210	164	95	136
Feces			0.06	0.04				
Urine	0.10	1.21	2.08	1.39				
Tail	1.58	2.4	1.93	1.99	131	154	169	244
Hair	0.62	1.48	1.43	1.22	100	125	114	166
Hide at inj. site			17.8	23.0			812	789
Remainder of hide	18.2	19.0	13.6	14.4	225	233	247	343
Hind leg muscle	1.09	0.55	0.60	0.30	173	122	111	214
Remainder of rat	41.7	60.7	41.0	42.2	162	152	246	193
Total recovered	87	110	96	98				

* Rats A and B were injected intracardially and killed ½ hour later. Rats C and D were injected subcutaneously and killed 20 hours later. Tissues of rat A were extracted with trichloroacetic acid; those of rats B, C, and D were digested with alkali, and aliquots were precipitated with trichloroacetic acid.

TABLE 2

Concentrations of Sulfapyrazine in Serum, Tumor, Leg Muscle and Kidneys of 257 Rats

Dose of sulfa (mg/100 gm body wt)	Hrs. from sulfa inj. to killing	Concentration of sulfa in serum at killing (mg/100 ml)		Concentration of sulfa per gm. of fresh tissue divided by concentration per ml. of serum						Median tumor weight (gm.)	Median body wt. at killing (gm.)	No. rats
				Whole tumor		Leg muscle		Kidneys				
		Median	Third quartile	Median	Third quartile	Median	Third quartile	Median	Third quartile			
(Repeated injections of glucose)												
	25	86	91	0.77	0.78	0.40	0.41	1.02	1.03	9.4	330	2
75	46	86	92	0.82	0.83	0.40	0.42	0.81	1.07	4.6	192	3
	67	58	65	0.83	0.87	0.40	0.41	0.89	1.08	5.3	232	2
	25	101	109	0.86	0.90	0.50	0.53	0.82	0.84	1.5	323	4
	46	81	92	0.96	1.05	0.47	0.51	0.93	0.95	4.0	259	9
100	69	86	95	1.07	1.35	0.45	0.49	0.91	0.96	2.2	279	9
	97	30	30	1.29	1.45	0.31	0.31	0.92	0.92	5.6	179	1
	25	124	127	0.99	1.12	0.51	0.54	0.77	0.81	5.9	333	7
125	47	102	108	1.18	1.69	0.46	0.50	0.83	0.86	4.7	381	6
	69	87	89	2.75	3.14	0.55	0.62	0.96	0.97	4.5	406	3
150	25	143	152	1.56	1.80	0.57	0.58	1.25	1.28	3.9	309	2
200	22	206	211	1.08	1.33	0.49	0.51	0.89	0.93	2.4	346	4
Single injection of glucose												
200	20	181	191	0.82	0.96	0.48	0.58	0.99	1.15		313	25
400	5	275	391	0.51	0.91	0.41	0.48	0.63	0.75		317	7
600	4	229	304	0.96	1.43	0.50	0.80	0.81	1.28		269	30
	5	369	446	0.65	0.93	0.43	0.47	0.74	5.0		294	7
800	3	295	335	0.69	0.78	0.48	0.78	0.67	0.73		347	15
1,000	3	266	329	0.73	0.89	0.51	0.62	0.75	0.85		321	21
No glucose injected												
200	20	192	205	0.86	1.00	0.49	0.57	1.20	2.10		305	18
400	5	395	402	0.75	0.80	0.41	0.44	4.6	5.9		339	6
600	4	338	415	0.81	1.14	0.52	0.63	2.7	5.1		274	31
	5	445	536	0.80	1.19	0.43	0.60	6.3	10.0		256	7
800	3	337	399	0.73	0.85	0.39	0.53	1.5	2.9		344	17
1,000	3	363	397	0.91	1.20	0.53	0.65	1.6	3.7		318	21

centrations of sulfa in their kidneys and smaller kidneys (average 0.87 per cent of body weight, as compared to 0.99 per cent for the others).

A second experiment was then undertaken with glucose injections made twice daily in 52 rats. Most of these were given smaller doses of sulfapyrazine and were killed at intervals up to 4 days. Of the 90 tumors in these rats, 40 tumors in 31 rats (bearing 54 tumors) contained higher concentrations of sulfapyrazine, averaged for the whole tumor, than did serum from these rats. The median serum concentrations of sulfapyrazine were 96 mg/100 ml for the 31 rats and 97 mg/100 ml for the other 21 rats. Samples of necrotic material were taken for separate analysis from 27 of the 40 tumors and from 33 of the other 50 tumors. Of the 27 tumors, 23 had higher concentrations of sulfapyrazine in necrotic than in other portions analyzed, a condition also present in 28 of the 33 tumors. In thirteen of the 23 tumors, white flecks tentatively presumed to be precipitated sulfapyrazine were grossly visible, almost always in necrotic parts. Similar flecks were observed in seven of the 28 tumors. Their presence or absence, therefore, indicated correctly in about two out of three instances which whole tumors contained higher concentrations of sulfapyrazine than did serum. Pus found in seven infected tumors (of which three were also necrotic) contained higher concentrations of sulfapyrazine than did serum. In the 63 samples of non-necrotic tumor separately analyzed, sulfapyrazine concentrations varied directly with those in serum; a fairly constant proportion, 0.88 ± 0.11 (standard deviation), was maintained between non-necrotic tumor and serum over a wide range of concentrations, apparently unrelated to time. The highest distribution ratio observed in whole tumors was 3.3.

Distribution ratios for whole tumors varied both with tumor necrosis and with time after the injection of sulfapyrazine in a way that permitted only slight distinction between the two. The incidence of severely necrotic tumors increased with each successive day—52, 61, and 77 per cent—but only on the third day was there increased incidence of tumor distribution ratios exceeding unity (38, 36, and 62 per cent), with corresponding median distribution ratios of 0.95, 0.94, and 1.08.

Differences between tumors on a rat were as important in producing tumor distribution ratios exceeding unity as differences between rats. Tumor distribution ratios exceeded unity in only one of the two tumors on each of 28 rats, in nine of the nineteen tumors on them that were more than three-fourths necrotic. Of 38 similarly necrotic tumors in other rats, the same proportion —twenty tumors—had distribution ratios exceeding unity.

Nevertheless, there is evidence that rats with higher tumor distribution ratios differed from rats with lower ratios. The systematic variation of tumor distribution ratios with distribution ratios of other tissues was slight but definite. Thus, median distribution ratios for muscle, spleen, liver, testes, and heart were, respectively: 0.50, 0.56, 0.72, 0.48, and 0.71 for the seventeen rats with tumor distribution ratios exceeding unity; 0.46, 0.55, 0.70, 0.46, and 0.62 for the fourteen rats in which one tumor distribution ratio exceeded unity and the other did not; and 0.44, 0.52, 0.69, 0.46, and 0.63 for the 21 rats with tumor distribution ratios less than unity. The explanation for this is uncertain. Median serum concentrations were similar for these three groups of rats: 96, 96, and 97 mg/100 ml. Systematic variation occurred in body weights of these same three groups of rats: between sulfapyrazine injection and killing, nine of the seventeen rats, nine of the fourteen rats, and fifteen of the 21 rats lost weight. This suggests that we are concerned with physiological variation and not analytical artifact.

Precipitates and high local concentrations of sulfapyrazine were found in three locations other than tumors: the injection site showed visible precipitate whenever examined; the stomach in 34 of the 52 rats contained precipitate often accompanied by bleeding spots (median distribution ratios were 3.5, 1.4, and 1.2 at 1, 2, and 3 days, respectively, after sulfapyrazine injection); and precipitate was usually seen in kidney ducts, occasionally in ureters. In whole kidneys the concentration of sulfapyrazine only occasionally exceeded that in serum. The highest distribution ratios observed in kidneys and stomach, respectively, were 1.5 and 10.9.

In each of the other tissues rather constant proportions between tissue concentration and serum concentration of sulfapyrazine were found. The median distribution ratios at 1, 2, and 3 days, respectively, after sulfapyrazine injection were: hide, 0.96, 0.96, 0.92; blood, 0.85, 0.88, 0.92; urine, 0.83, 0.52, 0.51; liver, 0.76, 0.68, 0.66; lungs, 0.65, 0.65, 0.67; heart, 0.62, 0.65, 0.67; spleen, 0.56, 0.54, 0.56; leg muscle, 0.51, 0.45, 0.46; testes, 0.49, 0.46, 0.44. Two-thirds of these ratios lay within ± 0.1 of their respective medians, except those for urine, which lay within ± 0.2. Other tissues from these 52 rats were occasionally analyzed (number of samples in parentheses) and gave average tissue distribution ratios as follows: thymus (20), 0.70; thyroid (15), 0.71; ear pinna

(7), 0.89; ovaries (6), 0.58; small intestine (3), 0.86; contents of small intestine (2), 0.70; bone marrow (2), 0.60; pancreas (1), 0.53; and brain (1), 0.29.

COMMENTS

In evaluating these exploratory experiments, attention may be called briefly to several considerations. One kind of selective localization in cancer tissue of a compound administered at sites distant from the tumor has been produced. This localization may be tentatively considered the result of a number of factors, including acid precipitation. In the first experiment, lasting only a few hours, glucose administration did not affect the incidence of localization. Increased incidence was observed in the second, more prolonged experiment; localization occurred in half of the more necrotic tumors—whether it was influenced by glucose administration was not determined. Acid precipitation also presumably produced localization of the compound in stomach and in kidney ducts. The several animal test procedures that resulted in localization of sulfapyrazine in some tumors were generally lethal. Localization occurred almost entirely in the necrotic parts of tumors. Many tissues were not analyzed individually, so that either gross or microscopic localization other than that observed may have occurred. Principal sources of analytical error are thought to be the incomplete extraction of sulfapyrazine from tissues and the uncertain influence of sulfapyrazine metabolites, particularly in experiments with rats living several days after sulfapyrazine injection.

Aside from grossly localized sulfapyrazine, the distribution of sulfapyrazine among rat tissues resembled more nearly that of iodide (17) than that of chloride (11), although the tissue distribution ratios were higher for sulfapyrazine than for either of these halides. Exceptions were the testes (higher for chloride) and the thyroid and stomach (higher for iodide). The distribution ratios found for sulfapyrazine (pKa 6.04) were higher than those reported for sulfamerazine (pKa 7.06), sulfadiazine (pKa 6.48), or sulfaquinoxaline (pKa 5.9), which were for rat blood, 0.74, and for rat liver, 0.45 (15). They were also higher than those reported for sulfamerazine, sulfadiazine, and N^1-acetylsulfanilamide (pKa 5.38) in several tissues of cats (7). Both these similarities and differences remain to be explained. Protein-binding presumably had a large role, particularly evident in the serum concentrations of sulfapyrazine that were often several times those present in saturated aqueous solutions buffered with phosphate at pH 7.4.

SUMMARY

High local concentrations of sulfapyrazine in cancer tissue occurred after its injection at sites distant from the tumor. In rats given glucose injections twice daily, nearly half the tumors contained higher concentrations of sulfapyrazine than did serum. Concentrations were highest in the necrotic material. In other tissues concentrations were proportional to those in serum. They were usually lower than serum concentrations except in some stomachs, in kidney ducts, and at the injection site. The animal test procedures used were generally lethal. Subcutaneous implants of Walker tumor 256 were used; 295 tumors in 257 rats were analyzed.

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The Effects of Tumor Growth on the Ascorbic Acid Concentration of Mouse Organs*

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A relationship between ascorbic acid and tumor growth has been considered by many investigators (1). It has been demonstrated that this vitamin stimulated the growth of some tumors, depressed the growth of others, and had no influence on still other tumors. Much of this work has been carried out on the mouse, a species capable of synthesizing ascorbic acid. In these animals, the increased demand for ascorbic acid or the increased utilization of this substance may not be associated with any obvious deficiency symptoms. It seemed possible, however, that the observed variation in biological reaction might be associated with differences in tissue ascorbic acid concentration. The present study deals with the effects of transplantable tumors on the concentration of ascorbic acid in the tissues of the host.

MATERIALS AND METHODS

Five strains of mice from our own laboratory stock and a sixth, the C57L(F_1) hybrid, purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, were used in these experiments. Mice obtained from the breeding of C57L(F_2) hybrids were designated C57L(F_x) mice.

The tumors included MT-8, a mouse bronchogenic carcinoma (4); 755, a mouse mammary carcinoma; HC-330,[1] a hypernephroma from a human source; and the Brown-Pearce rabbit carcinoma (3). All tumors were transplanted subcutaneously into the axilla of mice of the appropriate strain. The mice were sacrificed 14–21 days after the transplantation of the tumors. Transfers of MT-8 and 755 were always successful, and tumors grew to a large size. The percentage of successful transplants of HC-330 was low, and the tumors that grew remained small. Brown-Pearce transplants grew in DBA mice, but in comparable periods of time they did not achieve the size of MT-8 or of 755 transplants.

Mice were anesthetized lightly with ether, bled by cardiac puncture, and dissected. Immediately upon dissection, the organs were placed in Petri dishes set in dry ice. Until analyses could be carried out, these were kept in sealed containers in a −60° C. refrigerator.

For the determination of ascorbic acid, the various tissues were weighed, extracted with cold 4 per cent trichloroacetic acid by grinding in a Ten Broeck tissue grinder which was kept in an ice bath, diluted to suitable volume, centrifuged, and then filtered. The analyses were carried out on aliquots of these filtrates by the method of Roe and Kuether (7). Analyses were done on individual livers and brains, but blood, adrenals, spleens and kidneys from four to six mice were pooled for more accurate analysis.

RESULTS

Table 1 contains a summary of the tissue levels of ascorbic acid in different strains of mice. Strain differences have been reported by other workers, and some are apparent here (2, 5). Except for a lower concentration of ascorbic acid in the kidneys of DBA mice, the differences in the concentrations of ascorbic acid in blood, liver, and brain were slight. An outstanding example of strain difference, however, was the concentration of ascorbic acid in the adrenal. The average values ranged from 87.2 mg/100 gm in the adrenals of C3H and C57BR mice to 231.5 mg/100 gm in C57BL mice. Leaden hybrids had adrenal values intermediate between these two. While most tissue ascorbic acid levels of C57BR mice were close to those of C57BL mice, the concentration in the adrenals of the brown mice was 37.7 per cent of that in the black mice. C57L(F_1) and F_x hybrids, which were originally derived from C57 strain mice, possessed high spleen ascorbic acid levels similar to C57 black and brown mice.

The ascorbic acid levels in comparable organs of DBA and C3H mice paralleled one another closely but were generally lower than the same organs from C57 mice. The most significant differences appeared to be the lower concentrations of ascorbic acid in adrenal and spleen.

The effects of subcutaneous transplantation of tumors on blood and tissue ascorbic acid levels are shown in Table 2. The ascorbic acid levels in all tissues of tumor-bearing DBA and C3H mice were the same or higher than those in comparable normal mice. Increases of the greatest magnitude occurred in the kidneys and livers of the DBA mice.

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[1] Tumor HC-330 was obtained from Prof. Harry S. N. Greene as a growth from the anterior chamber of the guinea pig eye.

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TABLE 1

TISSUE ASCORBIC ACID LEVELS IN NORMAL MICE OF DIFFERENT STRAINS (MG/100 GM WET TISSUE)

Strain	Blood	Adrenal	Spleen	Liver	Kidney	Brain
DBA	0.94±0.07*	127.4±12.4	34.4±2.21	26.2±1.60	11.8±0.92	50.4±1.06
C3H	1.13±0.05	87.2± 9.49	37.4±3.34	27.5±1.85	14.8±0.43	52.0±1.46
C57BL	1.27±0.06	231.5± 8.41	45.7±3.22	30.7±1.59	19.1±0.99	54.2±0.94
C57BR	1.12±0.08	87.2±17.5	43.9±1.75	25.2±0.46	16.5±0.61	56.4±1.08
C57L (F_1)	1.19±0.02	155.0±11.5	49.2±1.57	27.4±1.73	19.1±0.77	56.2±1.02
C57L (F_x)	0.98	153.9	48.7	26.5±0.55	16.2	53.3±0.91

* Standard error of the mean.

TABLE 2

TISSUE ASCORBIC ACID LEVELS IN MICE WITH TUMORS

Mouse strains	Blood (mg/100 ml)	P*	Adrenal (mg/100 gm)	P	Spleen (mg/100 gm)	P	Liver (mg/100 gm)	P	Kidney (mg/100 gm)	P	Tumor (mg/100 gm)
DBA:											
Normal (no tumors)	0.94±0.07†		127.4±12.4		34.4±2.21		26.2±1.60		11.8±0.92		
With MT-8 transplants	1.48±0.23	0.05–0.02	169.7±22.4	0.2–0.1	36.3±0.68	>0.5	37.0±2.24	0.01–0.001	18.4±1.31	0.01–0.001	18.1±2.7
With Brown-Pearce transplants	1.16±0.08	0.1 –0.05	127.4±27.2	N.C.	35.6±1.93	>0.50	37.2±1.63	<0.001	18.4±2.46	0.02–0.01	35.9±3.3
With HC-330 transplants	1.59	‡	173.9		38.3		33.2		17.3		
C3H:											
Normal (no tumors)	1.13±0.05		87.2± 9.5		37.4±3.34		27.5±1.85		14.8±0.43		
With MT-8 transplants	1.17±0.13	>0.5	124.2±14.5	0.1–0.05	39.2±3.48	>0.5	35.4±4.40	0.2–0.1	17.4±0.66	0.01–0.001	21.0±1.1
With spontaneous mammary tumors			258.8	<0.001	33.4	>0.5	25.9	>0.5	18.7	0.02–0.01	43.4±7.6
C57BL:											
Normal (no tumors)	1.27±0.06		231.5± 8.4		45.7±3.22		30.7±1.59		19.1±0.99		
With 755 transplants	1.64		98.3± 8.1	<0.001	29.7	0.05–0.02	23.0	<0.001	9.2	<0.001	36.4±3.1

* Probability that deviation from normal could have occurred by chance; derived from the Student "t" test.

† Standard error of mean.

‡ Number of analyses done too few for statistical analysis.

N.C. = No change.

The adrenal levels were also often increased but with relatively wide variations. The increase of 42.2 per cent in the ascorbic acid content of the adrenals of C3H mice bearing MT-8 transplants takes on added significance, however, in light of the threefold increase observed in the adrenals of C3H mice bearing spontaneous mammary carcinomas. Adrenal ascorbic acid levels in DBA mice bearing MT-8 transplants were also increased, as were those in the group bearing HC-330. Brown-Pearce tumor transplants did not exert this effect.

The ascorbic acid levels of C57BL mice, which normally possessed the highest tissue levels, were affected differently by tumor transplants than the other two strains of mice. Except for the increased blood level of mice bearing tumor 755, the over-all *decrease* in the ascorbic acid concentrations of all tissues analyzed was of considerable magnitude.

The content of ascorbic acid in the different tumors varied. The tumor containing the lowest ascorbic acid concentration was MT-8, and the tumor containing the highest concentration was the spontaneous mammary tumor. There was no apparent correlation between the level of ascorbic acid in the tumor and in the tissues. Growth in DBA or C3H strain mice did not alter the ascorbic acid concentration in MT-8.

DISCUSSION

It does not appear that changes in the hosts' stores of ascorbic acid were directly associated with the nutritional demands of the tumor, for the extent of depletion or of increased production of the vitamin could not be correlated with the growth rate or size of the tumor. Tumor 755 grows as rapidly in C57BL mice as MT-8 grows in DBA or C3H mice, yet tumor 755 induced a generalized decrease in the ascorbic acid of the hosts' tissues, while the growth of MT-8 lead to increased levels.

The alteration in tissue ascorbic acid levels associated with tumor growth might have been an indirect response to nutritional or metabolic requirements of the tumor. Thus, the Brown-Pearce tumor produced increases in the ascorbic acid content of the kidney and liver but not of the adrenal. Growth in DBA mice was slow, and the tumors remained small. Greene (3) has reported that this tumor can also be transplanted to normal C3H mice but that takes are rare and growths small. In C3H mice bearing spontaneous mammary tumors, however, he obtained 100 per cent takes, and the tumors grew to a large size. In the present experiments, the adrenals of C3H mice bearing spontaneous mammary tumors contained very high concentrations of ascorbic acid. It is possible that the presence of increased amounts of ascorbic acid in the adrenal is an indicator of changes in other factors that influence the tumor directly. From the work of Sayers *et al.* (8) and Long (6), it is now acknowledged that the level of ascorbic acid in the adrenal and possibly in other tissues is controlled by hormones from this gland and from the pituitary. The variations in tissue ascorbic acid that occur during the growth of tumors may, therefore, be the result of alterations induced in pituitary-adrenal balance.

SUMMARY

The ascorbic acid levels of normal organs of six strains of mice were compared. The concentration in the spleens of C57 mice and in related strains appeared to be higher than in DBA or C3H mice. The concentration of ascorbic acid in the adrenals of C57BL mice was the highest of the six strains examined. The concentration of ascorbic acid in the kidneys of DBA mice was lower than in the five other strains. The amounts in blood, liver, and brain were similar in all six strains.

Increases of varying degrees were observed in the ascorbic acid concentrations in kidneys and livers of DBA and C3H mice bearing transplants of a mouse bronchogenic carcinoma, the Brown-Pearce rabbit carcinoma, or a human hypernephroma. Adrenals as well as kidneys of C3H mice bearing spontaneous mammary carcinomas possessed significantly increased amounts of ascorbic acid. In C57BL mice, transplants of mammary carcinoma 755 induced decreases in tissue ascorbic acid levels.

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The Effects of the Administration of Ascorbic Acid and of Rutin on the Transplantability of a Hepatoma and on the Ascorbic Acid Levels of Mouse Organs*

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Hepatoma C954 calls forth a marked vascular response and has a limited host range.[1] Since ascorbic acid plays a role in the metabolism of vascular tissue, the effect of injected ascorbic acid on the growth of this tumor in resistant strains of mice was considered. To enhance the action of ascorbic acid, rutin was also administered to certain animals (1, 3).

MATERIALS AND METHODS

Hepatoma C954 was obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and was maintained in C57L(F_1) mice from this source, prior to transfer to other strains. Tumor from these mice was transferred subcutaneously into the axilla of C57BL, C57BR, DBA, and C57L(F_x) mice (obtained from the breeding of F_1 hybrids).

In preliminary experiments, the administration of ascorbic acid in an oily base effectively maintained a prolonged high blood level of ascorbic acid. Therefore, stable emulsions of the substances to be injected were prepared by mixing equal volumes of their aqueous solutions with Pendil, a commercial preparation of peanut oil, beeswax, and oxycholesterol. Mice were injected intramuscularly with 0.1 ml. of the oily suspensions containing saline solution, sodium ascorbate (25 mg/ml), rutin[2] (25 mg/ml), or a mixture of ascorbic acid and rutin, respectively. Injections were started at the time of implantation of the hepatoma and were repeated on alternate days. Tumor incidence and latent period were recorded. The preparation and extraction of the tissues prior to the determination of their ascorbic acid content was described in the preceding paper (2).

RESULTS

Transplantation of hepatoma C954.—Of forty transplants into C57L(F_1) mice, 38 hepatomas grew successfully. A small mass became palpable in 9–14 days and continued to grow rapidly, especially in a direction parallel to the long axis. Transplantation in the C57L(F_x) mice was less successful, and only 34.6 per cent of the transplants grew. Growths were palpable in from 8 to 14 days; they had the same gross and microscopic characteristics and ran the same course as tumors in F_1 hybrids. No growths resulted in twelve C57BL or in ten C57BR mice. In eight of the fourteen DBA mice into which transfers were made, very small palpable growths appeared in 10–12 days, but these remained small and regressed about 4 days later.

The effects of the administration of ascorbic acid, or rutin, and of a combination of ascorbic acid and rutin on the growth of hepatoma C954 can be seen in Table 1. In C57L(F_1) hybrids injected with ascorbic acid, tumors were palpable 18–21 days after they were implanted. In the control groups, the latent period varied from 9 to 12 days. There were no differences in the number of successful transplants, in the gross and microscopic appearance, or in the subsequent course of the tumors in these two groups. This was also true for the group treated with the combination of ascorbic acid and rutin, although palpable tumors were frequently noted on the eighth day after transfer.

In C57L(F_x) hybrids, the incidence of tumor takes rose from 35 per cent in the control group treated only with the oily suspension of saline, and 37 per cent in the group that was treated with rutin, to 52 per cent in the group that received ascorbic acid, and 80 per cent in the group injected with the combination of ascorbic acid and rutin. Tumors in this last group also appeared to have a slightly shorter latent period.

Ascorbic acid alone, rutin alone, or the combination of these substances were without effect in altering the resistance of DBA and C57BR mice to hepatoma growths. In the group of six C57BL mice that was treated with ascorbic acid and rutin, there were four growths of hepatoma which be-

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[1] Personal communication from Dr. Elizabeth S. Russell, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

[2] Rutin used in these experiments was very kindly furnished by the Squibb Co., New York, N.Y.

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came palpable 19 days after transfer. Transplants did not grow in any of the other groups of C57BL mice.

Tissue levels of ascorbic acid.—The ascorbic acid concentrations in the tissues of normal and tumor-bearing C57L(F_1) hybrids are recorded in Table 2. The growth of hepatoma transplants in F_1 mice appeared to have little or no effect on the ascorbic acid concentration in the adrenal or liver. Tumor growth brought about a reduction of about 15 per cent in the blood, a smaller decrease in the brain, and a decrease of 21 per cent in kidney ascorbic acid concentration. The greatest decrease (35 per cent) occurred in the ascorbic acid of the spleen. The probability that such a decrease could have occurred by chance is less than one in a thousand.

Following treatment with ascorbic acid, there was an increase in the ascorbic acid content of the blood, kidney, and liver. The administration of ascorbic acid, however, did not prevent a decreased concentration in the spleens of tumor-bearing F_1 hybrids.

Blood levels of ascorbic acid in C57L(F_1) mice with tumors were increased to the same extent in the groups treated with both ascorbic acid and rutin as in the group treated with ascorbic acid alone. An increase in the ascorbic acid content of the adrenal, however, was observed only in the groups treated with the combination of rutin and ascorbic acid. The ascorbic acid levels in tumors from both groups that received ascorbic acid were 20 per cent higher than in tumors from untreated groups. The administration of these substances did not prevent the decrease in spleen ascorbic acid concentration observed in tumor-bearing mice. This decrease of 38 per cent is statistically significant.

The tissue ascorbic acid levels of C57L(F_x) mice are recorded in Table 3. The levels in normal organs were the same as in the F_1 hybrids. In untreated F_x hybrids, growth of tumors was associated with little change in tissue ascorbic acid concentration. In those mice treated with ascorbic acid, there was a decrease in the ascorbic acid content of the spleen when tumors grew. This de-

TABLE 1

THE EFFECT OF ASCORBIC ACID AND RUTIN ON THE TRANSPLANTABILITY OF HEPATOMA C954

Strain of mice	No. injections of 0.1 ml.* (Oil suspensions)				Takes/no. of transplants	Latent period (days)
	Saline	Ascorbic acid	Rutin	Ascorbic acid and rutin		
C57L (F_1)					23/25	9–14
	2				10/10	10–12
	6				5/5	10–12
					38/40 (total)	
		4			6/6	21
		6			4/5	18
					10/11 (total)	
				2	6/6	8–13
				4	3/3	8–13
				6	6/6	9–11
				9	6/6	8–13
					21/21 (total)	
C57L (F_x)					4/10	8–12
	2				2/10	12–13
	6				3/6	10–13
					9/26 (total)	
		2			5/6	10–12
		3			3/10	12–14
		6			6/8	14–18
		10			4/10	10–12
					18/38 (total)	
			2		6/7	9–11
			6		2/10	10–12
			10		2/10	10–13
					10/27 (total)	
				2	6/6	8–10
				3	9/10	8–10
				4	5/8	8–9
				5	6/7	11–13
				6	6/6	6–10
				8	3/9	9
				10	10/10	9–12
					45/56 (total)	

* Ascorbic acid suspension contained 25 mg/ml.
Rutin suspension contained 25 mg/ml.
Rutin and ascorbic acid suspension contained 25 mg/ml of each.

creased level was observed in animals killed 24 hours and 1 week after the last injection of ascorbic acid. In another experiment, this lowered ascorbic acid concentration in the spleen of tumor-bearing C57L(F_x) mice was observed as long as 6 weeks after the last injection of ascorbic acid. The spleens of two tumor-bearing mice in the rutin-treated group did not show this decrease. In resistant mice, the ascorbic acid content of the spleen was either normal or elevated. The average spleen level in all C57L(F_x) mice in which hepatomas grew was 34.0 ± 3.15 mg of ascorbic acid/100 gm of wet tissue; in spleens of resistant mice in all groups it was 56.1 ± 2.90 mg/100 gm of tissue. The probability that such a difference could have occurred by chance is less than one in a thousand. While the highest ascorbic acid concentration of the spleen (65.8 mg/100 gm) occurred in the one resistant mouse that was treated with ascorbic acid and rutin, the average ascorbic acid level of the spleens of eight resistant mice in the control group injected with only the oil-saline suspension was similar (64.4 mg/100 gm).

The ascorbic acid content of the livers of mice in all groups in which hepatomas grew was slightly higher than normal. When implanted hepatomas failed to grow, the administration of ascorbic acid or rutin gave rise to definitely increased levels in the liver. The ascorbic acid content of the adrenals in treated and untreated C57L(F_x) mice was variable.

Table 4 shows the results of an experiment with C57BL mice. There were four groups, consisting of six mice in each group. All received subcutaneous

TABLE 2

ASCORBIC ACID LEVELS IN TISSUES OF C57L (F_1) MICE

	Blood (mg/100 ml)	Adrenal (mg/100 gm*)	Spleen (mg/100 gm)	Liver (mg/100 gm)	Kidney (mg/100 gm)	Brain (mg/100 gm)	Hepatoma (mg/100 gm)
Normal Mice	1.19±0.02†	155.0±11.5	49.2±1.57	27.4±1.73	19.1±0.77	56.2±1.02	
Mice with hepatoma transplants for 14–18 days	1.01±0.03 (0.01–0.001)‡	162.7±30.5 (>0.5)	31.8±0.63 (<0.001)	30.4±2.68 (0.5–0.2)	15.1±1.20 (0.05–0.02)	47.8±3.08 (0.05–0.02)	8.5±0.34
Mice with hepatoma transplants also injected with ascorbic acid§	1.48±0.04 (0.05–0.02)	108.7±29.9 (0.5–0.2)	28.9±1.43 (<0.001)	40.1±1.84 (0.01–0.001)	31.2±2.01 (0.02–0.01)		10.1±0.52
Mice with hepatoma transplants also injected with ascorbic acid and rutin§	1.52±0.10 (0.05–0.02)	237.2±36.5 (0.1–0.05)	30.7±1.00 (<0.001)	29.5±2.68 (>0.5)	19.2±1.21 (N.C.)#	51.1±2.61 (0.2–0.1)	10.4±0.94

* Wet tissue.
† Standard error of the mean.
‡ Probability that deviation from normal could have occurred by chance.
§ Animals sacrificed two weeks after the last injection.
# No change.

TABLE 3

ASCORBIC ACID LEVELS OF TISSUES OF C57L (F_x) MICE

Treatment of mice	Time of sacrifice*	Blood (mg/100 ml)	Adrenal (mg/100 gm†)	Liver (mg/100 gm)	Kidney (mg/100 gm)	Spleen (mg/100 gm)	Tumor (mg/100 gm)
Normal controls		0.98	153.9	26.5	16.2	48.7	
Received hepatoma transplants:							
Transplants grew							
Injected with saline‡	24 hrs.			32.7	16.2	45.3	27.0
Injected with ascorbic acid‡	24 hrs.			31.5	18.1	29.6	18.5
Injected with rutin‡	24 hrs.		293.1	39.7	17.5	44.1	16.4
Injected with ascorbic acid and rutin	24 hrs.		123.8	31.2	15.5	31.0	11.3
Injected with ascorbic acid	1 wk.	1.14	118.9	27.3	13.1	28.4	8.2
Injected with ascorbic acid and rutin	1 wk.	1.48	172.1	35.7	16.0	25.7	8.7
Injected with ascorbic acid	6 wks.		140.0	29.6	11.0	26.8	3.2
Transplants did not take							
Injected with ascorbic acid	24 hrs.	0.99	151.2	42.9	15.4	55.1	
Injected with rutin	24 hrs.	1.62	149.4	45.9	36.8	45.8	
Injected with saline	1 wk.		75.4	30.2	12.1	64.4	
Injected with ascorbic acid	1 wk.	0.83	286.3	44.0	19.7	54.5	
Injected with rutin	1 wk.	1.14	163.7	42.4	14.6	50.6	
Injected with ascorbic acid and rutin	1 wk.		72.1	43.3	22.8	65.8	

* Time after the last injection.
† Weight of wet tissue.
‡ All materials were injected as oil suspensions. Ascorbic acid and rutin preparations contained 25 mg/ml.

transfers of hepatoma. One group received the oily suspension of saline, the second received ascorbic acid, the third received rutin, and the fourth the combined rutin and ascorbic acid in the same vehicle. Each group was given nine injections on alternate days. In the fourth group, 21 days after the transfers were made, there were four palpable tumors. The animals were all sacrificed 10 days after the last injection and the tissue content of ascorbic acid determined. In the fourth group, the tissues of only tumor-bearing mice were analyzed. In all mice, the levels of ascorbic acid in the liver and brain remained unchanged. Adrenal ascorbic acid was somewhat decreased in the oil-saline suspension and rutin groups, even though implanted tumors did not grow. In the rutin–ascorbic acid-treated group, the ascorbic acid concentrations in the adrenal, kidney, liver, and brain of mice with hepatomas were normal or slightly elevated, while the spleen levels in these mice were lower than normal. The concentrations of ascorbic acid in the spleens of resistant mice in the three other groups were higher than in normal spleens, just as in resistant C57L(F_x) mice.

TABLE 4

ASCORBIC ACID LEVELS OF TISSUES OF C57 BLACK MICE

MICE	Ascorbic acid (mg/100 gm wet tissue)					
	Blood	Adrenal	Spleen	Liver	Kidney	Brain
Normal controls	1.27	231.5	45.7	30.7	19.1	54.2
Received hepatoma transplants* and:						
Saline†	1.19	189.5	57.6	28.6	19.4	58.0
Ascorbic acid†	0.92		51.7	31.7	8.2	56.5
Rutin†	1.26	187.1	47.8	35.7	14.6	52.8
Ascorbic acid and rutin†	1.06	276.3	40.6	33.5	22.4	55.3

* Transplants grew only in the groups treated with ascorbic acid and rutin.

† All materials were injected as oil suspensions. Ascorbic acid and rutin preparations contained 25 mg/ml.

DISCUSSION

The change in resistance to the growth of hepatoma transplants resulting from the administration of rutin and ascorbic acid does not appear to be brought about simply by the direct action of ascorbic acid. Their own endogenous supply of ascorbic acid, supplemented by rutin, did not alter the response of C57L(F_x) mice to hepatoma transplants. It would seem unlikely, therefore, that prolonging the action of ascorbic acid was the sole factor involved in the breakdown of tumor resistance. These substances did not appear to exert their influence directly on the transplant but did seem to affect particular organs which, in turn, might have influenced the growing tissue. For example, the pronounced decrease in spleen ascorbic acid during the growth of hepatoma transplants, even when there was an adequate exogenous supply, as well as the pronounced rise in the concentration of this substance in the spleens of mice in which transplants did not take, suggests a link between the metabolic activities of this organ and the fate of tumor transplants.

The fact that rutin and ascorbic acid did not alter the resistance of DBA and C57BR mice to the growth of hepatoma transplants is further evidence that these substances did not influence the tumor directly. At least in C57L mice there appeared to be a modifying influence necessary for the growth of hepatomas which was also present in F_1 hybrids but which was lost in a large per cent of their progeny. This influence appeared to be restored by the administration of rutin and ascorbic acid. Whether the administration of these substances merely replaced a necessary intermediate or provided a specific stimulus is unanswered by these experiments.

SUMMARY

The administration of ascorbic acid, combined with rutin, increased the number of takes of hepatoma transplants in a resistant group of leaden hybrids from 35 to 80 per cent. The resistance of C57BL mice to this tumor was also decreased by this combination of substances. The administration of ascorbic acid alone or of rutin alone was without effect.

In mice in which hepatoma transplants grew, there was a marked decrease in the ascorbic acid concentration in the spleen, even when additional ascorbic acid was given to the animal. In mice which resisted the growth of the hepatoma, the level of ascorbic acid in the spleen was high.

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The Effects of Dietary Riboflavin and Pantothenic Acid on the Metabolism of 2-Aminofluorene*

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The carcinogen 2-acetylaminofluorene, when fed to animals (0.03 per cent in the diet), produces a riboflavin deficiency and depletes protein stores, changes which can be prevented in part by increasing the riboflavin and protein content of the diet (1, 4, 5, 14). The following studies are part of a program of research on the metabolism of carcinogens, with observations on the effect of dietary riboflavin and pantothenic acid on the conjugation of 2-aminofluorene and on the formation of nonvolatile phenols in dogs.

MATERIALS AND METHODS

Adult beagle dogs (2–3 years of age, 8–10 kg.) were fed the synthetic diet previously reported (1). The carcinogen and the additional vitamins were administered orally in gelatin capsules. The carcinogen was administered after the animals had been on the test level of riboflavin or pantothenic acid for 7 days. Urine was collected for 3 days following administration of the carcinogen.

Aminofluorene and diazotizable amino groups were determined by diazotization followed by coupling with the *o*-toluidide of 2-hydroxy-4-naphthoic acid (naphthol ASD).[1] The resultant stable red-orange dye was compared to a standard solution treated in the same manner. Diazotization and coupling after hydrolysis with 3 N HCl permitted an estimation of free and conjugated amino groups excreted in the urine. The details of the analysis are as follows: 1 ml. of 3 N hydrochloric acid and 1 ml. of 0.03 N sodium nitrite were added to 1 or 2 ml. of urine (containing at least 8 μg/ml of the carcinogen). After mixing and waiting 1 minute, 2 ml. of freshly prepared 0.01 M naphthol ASD in 2.5 M NaOH were added, slowly, but with rapid agitation of the mixture. The volume was brought to 25 ml. with water and the reaction allowed to proceed for 30 minutes. The optical density was read at 530 mμ, the peak of the absorption curve (Chart 1). Blank determinations were made routinely in normal urine. The color was always negligible. The quantity of diazotizable amine was computed from a standard curve prepared with known amounts of 2-aminofluorene. A linear relationship was observed between concentration and optical density. The conjugated amine was determined by adding 2 ml. of the 3 N HCl to 2 ml. of the unknown and heating for 1 hour at 100° C. The base so liberated was determined as above. This is a modification of a method previously reported by Westfall and Morris (15).

Urinary nonvolatile phenols (NVP) were determined by the method of Valterra (13), results being reported as mg. phenol.

RESULTS AND DISCUSSION

Nutritional studies in our laboratories have indicated that 0.025 mg riboflavin/day/kg of body weight is an adequate intake of this vitamin for adult dogs. Animals fed this amount of vitamin in the presence of aminofluorene, however, do not conjugate a maximum amount of diazotizable amine. The data in Table 1 illustrate the increase in conjugation as the dietary riboflavin is raised from 0.025 to 0.25 to 2.5 mg/day/kg of body weight. These data were obtained by feeding the test level of vitamin for 7 days prior to administration of the carcinogen. Continued feeding of 2.5 mg. of riboflavin for 16 days increased the percentage of conjugation (expressed as conjugated aminofluorene) still further to an average of 51. Riggs and Hegsted (10) found that the conjugation of *p*-aminobenzoic acid was significantly lower in riboflavin-deficient rats than in the controls. On the other hand, Shils *et al.* (11) did not find riboflavin to be a factor in the acetylation of sul-

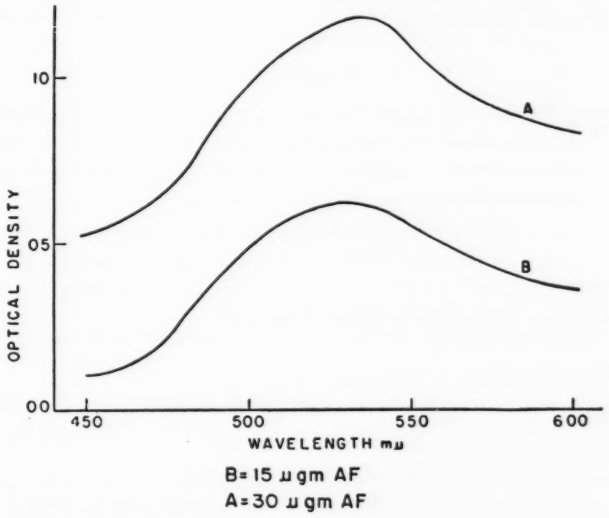

CHART 1.—Visible absorption spectrogram of the diazo compound of 2-aminofluorene and naphthol ASD.

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[1] The sample of naphthol ASD was generously supplied by R. Erdmann, Sr., of the Sinclair Valentine Company, N.Y.

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fanilamide. The latter authors point out that conjugation may be related to liver size, a factor which can be effected by riboflavin intake. These papers, however, emphasize the importance of pantothenic acid in conjugation reactions.

This increased conjugation of 2-aminofluorene was established in the presence of a constant amount of pantothenic acid in the diet (0.2 mg/day/kg body weight), a vitamin which has an effect upon conjugation of amines (9, 10, 12). The experiments were repeated, therefore, varying the pantothenate as well as the riboflavin content of the diet. The data in Table 2 illustrate again the increased conjugation of diazotizable amines associated with a rise in riboflavin intake. These data demonstrate also that increasing the pantothenate content of the diet raised the level of conjugation in the urine, an effect which was minimal in the presence of relatively large amounts of riboflavin. Thus, conjugation of the carcinogen is associated with the dietary level of both pantothenic acid and riboflavin. These data emphasize the variation in vitamin requirements of animals according to the stress placed upon them. The significance of these findings in terms of activity of enzyme systems is under investigation. It is possible that the dietary riboflavin, as a component of particular enzyme systems, forces the equilibrium in the direction of increased conjugation, a reaction which is associated with pantothenic acid. It is also possible that increased synthesis of liver protein, associated with adequate riboflavin (1), is a factor in developing maximum function.

Experience with 2-aminofluorene in dogs indicates that the conjugation is initially greater with aminofluorene than with the acetylated derivative. Six dogs, for example, were fed single 1-gm. doses of 2-acetylaminofluorene, and the amounts of the free and conjugated material excreted were measured. The percentage of conjugated carcinogen excreted versus time is illustrated in Chart 2. One-gm. doses of the aminofluorene hydrochloride were also fed, and the percentage of conjugation was measured. These data demonstrate that dogs fed the acetyl derivative excreted a constant percentage of the conjugated form, whereas those fed the hydrochloride excreted a high percentage initially —a percentage which decreased rapidly during the

TABLE 1

THE RELATION OF DIETARY RIBOFLAVIN TO EXCRETION AND CONJUGATION OF DIAZOTIZABLE AMINO GROUPS IN DOGS FED 2-AMINOFLUORENE HYDROCHLORIDE AND 2-ACETYLAMINOFLUORENE

The results are expressed as conjugated aminofluorene. The normal diet contained 0.025 mg of riboflavin/day/kg of body weight. The higher riboflavin intakes were established 7 days before feeding the carcinogen. Average of data obtained on three dogs.

Carcinogen	2-acetylaminofluorene			2-aminofluorene		
Riboflavin intake (mg/day/kg body weight)	0.025	0.25	2.5	0.025	0.25	2.5
Carcinogen intake (mg/day)	1,273	1,273	1,273	316	316	316
Urinary carcinogen (mg/day)	72	100	97	22	30	33
Per cent conjugated in urine	54(51–56)	62(49–66)	74(67–80)	7.2(5–9.7)	18(10–30)	39(24–53)

TABLE 2

THE EFFECT OF VARYING DIETARY PANTOTHENATE AND RIBOFLAVIN ON THE PER CENT CONJUGATION OF DIAZOTIZABLE AMINES IN THE URINE OF DOGS FED 2-AMINOFLUORENE HYDROCHLORIDE

(The carcinogen intake was 200 mg/day)

	Average of data on three dogs		
Calcium pantothenate intake (mg/day/kg body weight)	0.2	2.0	20.0
Experiment A: (0.025 mg riboflavin/day/kg) Per cent urinary amine conjugated	2.7	12.3	20.1
Experiment B: (2.5 mg riboflavin/day/kg) Per cent urinary amine conjugated	55.3	56.0	59.1

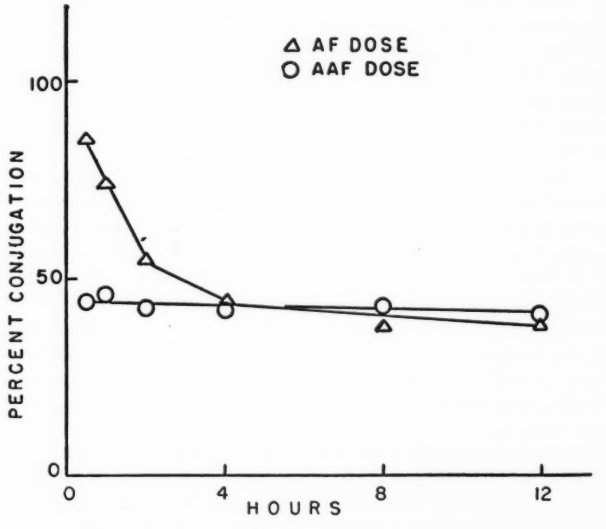

CHART 2.—Per cent of conjugated carcinogen excreted in the urine after single 1-gm. doses of 2-aminofluorene and 2-acetylaminofluorene. Average results with six dogs for each point.

first few hours to values obtained from animals fed the acetyl derivative. Deacetylation of 2-acetylaminofluorene has been demonstratad by Morris, Weisberger, and Weisberger (6). Thus, a dynamic equilibrium between deacetylation and conjugation could be established in the animal. It will be necessary to determine the chemical nature of the compounds excreted in the urine and labeled diazotizable amine before the significance of this dynamic equilibrium can be explored further. Data have also been obtained to demonstrate conjugation of β-naphthylamine in the dog (2), a conjugation which is a function of the pantothenic acid content of the diet.

2-Aminofluorene is carcinogenic in the rat (7) and is more toxic than the acetylated derivative in the dog. Dogs fed large amounts of the 2-acetylaminofluorene showed no clinical abnormalities; but those fed the amine exhibited marked cyanosis, impaired respiration, and methemoglobin formation, effects which began to disappear 3 hours after the administration of 1 gm. of the hydrochloride. It is possible, as suggested by Ray and Argus (8), that conjugation is a step in detoxification reactions.

The data in Table 3 illustrate the larger than normal excretion of nonvolatile phenols in the presence of the carcinogen and a high riboflavin intake. It is possible, therefore, that excess riboflavin has the general effect of increasing the metabolism of 2-aminofluorene resulting not only in an increased conjugation but also in hydroxylation. Beilschowsky (3) has demonstrated that a hydroxylated compound is the principal metabolite of aminofluorene in the rat. The protective effects of riboflavin could be the result of increased detoxification of the carcinogen through various metabolic pathways.

TABLE 3

NONVOLATILE PHENOL (N.V.P.) EXCRETION OF THE DOG IN RELATION TO AMINOFLUORENE AND RIBOFLAVIN

Dog no.	2-Aminofluorene	Riboflavin intake (mg/day/kg body weight)	N.V.P. excreted (mg/day/kg body weight)
147	none	2.5	2.7
158	none	2.5	2.9
159	none	2.5	2.3
87	316 mg.	0.025	4.5
		0.25	5.8
		2.50	6.5
106	316 mg.	0.025	4.1
		0.25	5.9
		2.50	7.2
141	316 mg.	0.025	4.4
		0.25	5.2
		2.50	6.0

SUMMARY

A modified method of analysis for 2-aminofluorene is described. This method involves diazotization followed by coupling with the *o*-toluidide of 2-hydroxy-4-naphthoic acid to form a stable dye. Conjugation was determined by liberating amine with acid hydrolysis.

A study was made of the effects of dietary pantothenic acid and riboflavin on the conjugation of diazotizable amine in the urine of dogs fed 2-aminofluorene. It was found that raising the level of dietary riboflavin increases the excretion of the conjugated amine. Conjugation is also a function of the pantothenic acid intake, being reduced by a pantothenic acid deficiency. The data demonstrate that dogs fed the acetyl derivative of 2-aminofluorene excrete a constant percentage of the conjugated form, whereas those fed the hydrochloride excrete a high percentage initially, a percentage which decreases rapidly to values obtained in animals fed the acetyl derivative.

The free amine is more toxic than the acetylated carcinogen, suggesting that conjugation is a form of detoxification—a reaction associated with the maintenance of adequate protein stores as well as with specific enzyme systems. Increasing dietary riboflavin increases the excretion of nonvolatile phenols in the dogs fed this carcinogen.

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The Action of Cortisone and ACTH on Transplanted Mouse Tumors

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The inhibition of the growth of normal (1) and malignant (5, 7) lymphocytes of mice and rats by cortical secretions is paralleled by the remissions obtained with cortisone and ACTH in human lymphomas (8). Some other types of tumors are inhibited in mice by maximal doses of these products (11) but are not affected in man by the tolerated amounts of the drugs (2). Early attempts of therapy against varied human tumors (12) prompted us to test the effect of ACTH and cortisone on two nonlymphoid tumors of mice. Experiments were also performed which demonstrated that these products do not modify the growth of Rous sarcomas induced in chickens by inoculation of the virus (4).

MATERIALS AND METHODS

The tumors were implanted in BALB/c mice obtained from the Jackson Memorial Laboratory and fed a commercial diet (Rockland Farms Pellets) supplemented with carrots.

The mammary carcinoma arose spontaneously in a mouse (B 324) of this strain. Two subcutaneous passages prior to this experiment resulted in 100 per cent takes. When the tumors reached a large size, they became in part necrotic or ulcerated. Metastases to the lungs and liver were frequent. The growth consisted of solid strands and cords of neoplastic cells occasionally forming acini or lining vascular spaces. The stroma was scanty.

For this experiment 0.1 ml. of a suspension of pooled mammary tumors in an equal volume of Locke's solution was injected under the skin of both flanks of eighteen 6-month-old female mice. One mouse of each group was injected in sequence. The size of the tumors was determined at intervals by palpation and plotted on a semi-logarithmic scale as the product of the three maximal dimensions. All mice were allowed to die.

The "ascites sarcoma" developed in mouse B 52 following an intraperitoneal implant of a pellet of paraffin impregnated with 0.5 mg. methylcholanthrene. After 101 days, the abdomen was distended by slightly bloody straw-colored fluid. A retroperitoneal nodule of fibrosarcoma, 8×5×5 mm., was also found at necropsy. The ascites was transferred by intraperitoneal injection of 0.4–0.8 ml. of ascitic fluid, with 22 takes out of a total of 25 mice injected in four passages prior to the present experiment. Small sarcomatous nodules were often found attached to the peritoneal surfaces or floating in the fluid. Injection of the fluid into soft tissues resulted in local fibrosarcomas. Smears of the ascitic fluid contained red blood cells, lymphocytes, macrophages, and a small number of round, presumably malignant cells isolated or in clumps; these cells varied in size and had dense nuclei.

For this experiment nine female 6-month-old mice each received intraperitoneal injections of 0.5 ml. of heparinized ascitic fluid 381. Eleven mice of the same age had injections of 0.5 ml. of pooled fluid 382 (from two mice of a different subtransfer of fluid 52). The mice were observed for abdominal swelling or tumors, and the diagnosis was confirmed at necropsy. Mice that developed no tumors were observed for 8 months.

Intramuscular injections of cortisone acetate (Merck) or of ACTH (Wilson) were given daily until death (mammary carcinoma experiment) or until the 105th day. The treatment was started on the day of the implants at the "low level," 0.075 mg. of ACTH or 0.15 mg. of cortisone. At the "high level," 0.5 mg. of ACTH or 1 mg. of cortisone was injected daily only after tumors had developed (seventh day for the mammary tumor, 22d day for the ascites sarcoma). Groups of untreated mice were kept as controls.

RESULTS

All transfers of mammary carcinomas were successful and were fatal to the mice (Table 1). The tumors during their early stage followed an exponential rate of growth, and the untreated mice gained in total weight. As the tumors became necrotic or ulcerated, there was a flattening or even a recession of the growth curves, and the mice lost weight.

TABLE 1

EFFECT ON MAMMARY CARCINOMA

Treatment		Tumors Size* at 30 days			Av. surv.
	mg/day	No.	Av.	(range)	(days)
ACTH	0.5	6	3.0	(2.1–3.4)	45
ACTH	0.075	6	3.1	(2.6–3.6)	64
Cortisone	1.0	6	1.8	(1.1–2.3)	90
Cortisone	0.15	6	2.8	(2.5–3.2)	67
Control		12	3.2	(2.3–3.7)	52

* Log. of product of 3 dimensions in mm.

High levels of cortisone slowed the growth of the transplants and increased the survival time; the

* This work was conducted during the tenure by R. G. G. of a Public Health Service Special Research Fellowship of the National Cancer Institute. Present address: Veterans Administration Hospital, Houston 31, Tex.

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treated mice lost considerable weight before the implants became very large. With low levels of cortisone there was a lesser body weight loss, which was compensated by the bulk of the tumors; inhibition of the neoplastic growth appeared doubtful.

ACTH at both levels seemed without effect on the growth of the tumors or on the weight of the mice.

The results with the ascites sarcoma are given in Chart 1. The percentage of takes was higher and the latent periods shorter in transfers of ascites 381

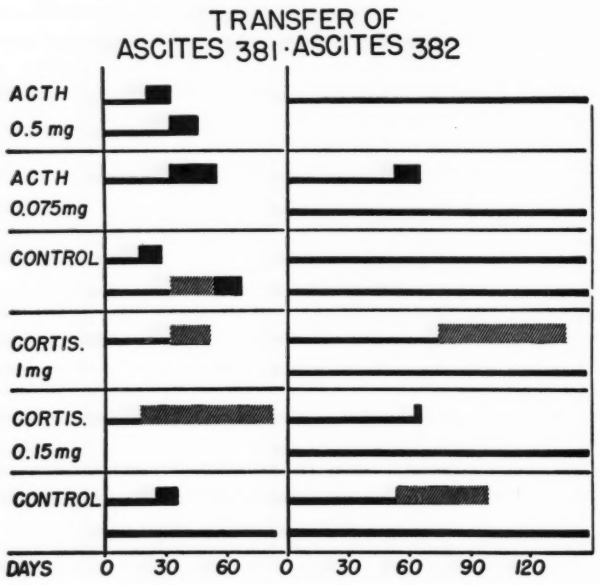

CHART 1.—Effect of ACTH and cortisone on transfers of ascites sarcoma in BALB/c mice.
Black fields: ascites with or without tumor nodules.
Cross hatched fields: tumor only.
Horizontal lines: no tumor or ascites.

than of ascites 382. The divided condition of the injected malignant cells and their prolonged contact with the peritoneum of the host during the latent period appeared favorable to chemotherapy. Nevertheless, ACTH and cortisone were without effect on the number of takes or on the latent periods of both subtransfers. Weight losses of the mice injected with cortisone were moderate at the low level, severe at the high level.

DISCUSSION

On a weight basis the lower amounts of ACTH and cortisone given to mice (about 3.75 and 7.5 mg/kg/day) were similar to the largest human therapeutic doses (100–200 mg/day of ACTH and 200–400 mg/day of cortisone); these are, however, too toxic for continued treatment (2, 3). The larger amounts of cortisone (about 50 mg/kg/day) are about the maximum dose tolerated by mice, reducing their food intake and producing marked anemia (11). Only at this high level was one of the two mouse tumors definitely inhibited, as were certain nonlymphoid mouse tumors reported in other studies (11).

The inhibition of the mammary carcinoma was probably not due to a physiologic balance, like the lymphoid-adrenal system, since comparable levels of ACTH did not affect the tumor. Divided doses of ACTH might, however, have been more effective. Cortisone is probably more "toxic" than the endogenous hormones released by corticotrophin.

Although cortical hormones inhibit fibroblasts in inflammatory reactions (9) and can decrease the reaction around various human tumors (3), they were ineffective in checking the growth of the ascites sarcoma and of various human sarcomas (2, 3).

The adverse effect of maximal amounts of cortisone on the mammary carcinoma and the weight loss of the mice appear to be nonspecific "toxic" effects. Both could, however, be the result of an increase of protein catabolism and gluconeogenesis (cf. 5), or of a general inhibition of growth, as observed by Karnofsky in chick embryos, newly hatched chicks, and baby mice, with high levels of cortisone or smaller amounts of Compound F (6, 11). Conversely, an increase of body weight and an acceleration of growth of a transplanted mammary carcinoma have been recently observed in mice injected with pituitary growth hormone (10).

The inhibitory effect of cortisone on the growth of the mouse mammary carcinoma, observed in these experiments, is of no clinical significance, since it required the use of toxic doses of the drug.

SUMMARY

The rate of growth of a transplanted mouse mammary carcinoma was reduced by very large doses of cortisone, while the effect was doubtful at the usual human therapeutic level. ACTH did not affect this tumor. Neither product inhibited the growth of an ascites-producing sarcoma.

ACKNOWLEDGMENTS

The technical assistance of Mrs. Phyllis Swartz and of Mr. William A. Sellars is gratefully acknowledged. We are also indebted to Dr. Leo V. Curtin of Merck & Co. and to Dr. David Klein of Wilson & Co. for supplies of cortisone and ACTH used in the present study.

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Experimentally Produced Granulosa-Cell Tumors in Rabbits

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In an earlier publication (5) we reported production of granulosa-cell tumors in rats and rabbits. The complete data concerning these tumors in rabbits will now be presented, the tumors in rats having been discussed elsewhere (4). Young, adult rabbits (4.5–8.5 kg.) of mixed breeds were used. The method was similar to that used by M. S. and G. R. Biskind in rats (1). A portion of one ovary was transplanted to the splenic or intestinal mesentery of the females after castration. In addition, a portion of ovary was transplanted in a similar manner into a group of male animals after they had been castrated.

RESULTS

Nineteen rabbits (thirteen ♀ and six ♂) were available for study. No viable grafts were found in the males (sacrificed from 19 to 652 days after transplantation). In the thirteen females[1] sacrificed after 11–673 days, eleven transplants were found, varying in age from 11 to 673 days. Four of these transplants will be excluded from further consideration, as they were obviously degenerating. The detailed data on the remaining seven appear in Table 1.

Three of the transplants could not be considered tumors, since relatively normal follicles and/or corpora lutea were present. The two younger were 399 (No. 29-2) and 491 (No. 30-2) days old and were grossly hemorrhagic. It was noted microscopically that the large follicles were filled with blood. Ova were present in many of the follicles. The third and oldest transplant (No. 21) was 673 days old. Grossly, it appeared pale yellow. Microscopic examination revealed normal follicles with ova. Corpora lutea were present and appeared normal, except that in some areas the architecture was disturbed and there were many granulosa cells scattered among the larger lutein cells.

There were no adhesions in the immediate vicinity of the transplants in these three animals. However, in one (No. 30-2) there were adhesions between the duodenum and parietal peritoneum. Its uterine horns and vagina showed microscopic evidence of minor estrogenic stimulation. These structures were atrophic in one of the others, and the findings were not recorded in the third.

The transplants from rabbits Nos. 51 and 55 were considered questionable tumors. In our preliminary report (5), one of these (No. 51) was considered a definite tumor. When re-evaluated, this transplant, as well as No. 55, was considered questionable.

Though devoid of normal structures and architecture, they were apparently degenerating. In some areas hyalinization and calcification were present. Other areas appeared relatively healthy and consisted of masses of luteinized cells arranged in small groups and cords separated by reticulum of varying density. Granulosa cells were inconspicuous and scattered about in small numbers. No adhesions were present in these animals, and there was no evidence of uterine or vaginal stimulation.

Two of the transplants (Nos. 27[2] and 22) were considered definite tumors. Grossly, they were yellow. No. 27 (Fig. 1) was made up of large luteinized cells mixed homogeneously with smaller granulosa cells. The luteinized cells contained copious amounts of eosinophilic, granular, and often vacuolated cytoplasm with spherical, large, and vesicular nuclei. These cells were often grouped with little or no intervening reticulum. The cell membranes were distinct. The granulosa cells were smaller, the nuclei were often irregular in outline with dense granular chromatin. Cell membranes were usually not visible, and each cell was surrounded by a fine network of reticulum. "Transition" forms between these two types of cells were frequent, suggesting that the large

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[1] Eight of these animals were mentioned in the preliminary report (5).

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[2] This tumor was described in the preliminary report (5).

lutein cells may have arisen from the granulosa cells. No adhesions were present in this animal, and the uterine horns and vagina were in a castrate condition.

The smaller tumor (No. 22, Fig. 2) was made up of partially luteinized cells with considerable cytoplasm but indistinct cell membranes arranged in cords and groups divided by relatively heavy reticular septa. Granulosa cells were present in lesser numbers, but there were none of the large lutein cells as in the previously described tumor. In some respects this tumor resembled adrenal tissue, but the individual reticular network surrounding each adrenal cell was not present in the tumor. No adhesions were present in the immediate vicinity of this tumor (the splenic mesentery), though the distal ileum was adherent to the parietal peritoneum. There was evidence of marked estrogenic stimulation both in the uterine horns (Figs. 3 and 4) and in the vagina.

TABLE 1

DETAILED DATA ON OVARIAN TRANSPLANTS IN CASTRATE RABBITS

Animal no.	Transplant age at sacrifice (days)	Size (mm. at greatest diam.)	Position of transplant	Character of transplant*	Location of adhesions	Condition genitalia
29-2†	399	4	Intestinal mesentery	Hemorrhagic follicles only	None	Not recorded
30-2†	491	2	Intestinal mesentery	Hemorrhagic follicles only	Duodenum to anterior abdominal wall	Slightly stimulated
51†	512	6	Intestinal mesentery	Predominately lutein cells, areas of degeneration	None	Atrophic
27†	533	6.4	Splenic mesentery	Definite tumor	None	Atrophic
22	597	2	Splenic mesentery	Definite tumor	Distal ileum to anterior abdominal wall	Highly stimulated
55	605	7	Intestinal mesentery	Mixed lutein and granulosa cells, areas of degeneration	None	Atrophic
21	673	10	Splenic mesentery	Normal follicles, corpora lutea	None	Atrophic

* See text for full description.
† These animals were referred to in the preliminary report (5).

DISCUSSION

It is generally agreed that granulosa-cell tumors develop in ovarian tissue transplanted into the portal circulation of castrate rats and mice as a result of prolonged, continuous gonadotrophic stimulation. The liver destroys the ovarian hormones, thus releasing the anterior pituitary from their usual cyclic inhibitory influence. In the guinea pig (3) such grafts at first contain numerous hemorrhagic follicles which are later replaced by extensive luteinization. In some of the oldest grafts this luteinization became extremely disorderly, but apparently in all there were either recognizable follicles or corpora lutea.

Our results with such transplants in rabbits are in some respects similar to those obtained in the guinea pig. In the two youngest grafts the "blood follicles" described by Lipschutz *et al.* were present (3). In our five oldest grafts, follicles and corpora lutea were found in only one (the oldest). In this graft the corpora lutea showed the disordered cell arrangement and "invasion" by cells which Lipschutz *et al.* believe to be distinct from granulosa cells (Figs. 5 and 6). Our interpretation has been that they are granulosa cells.

The apparent species difference with regard to the behavior of intrasplenic grafts between rats and mice, on the one hand, and guinea pigs on the other, may be only a difference in the time necessary for the development of such tumors. This contention (2) is strengthened by our observations in rabbits.

It is probable that some of these grafts were producing hormones, since in two animals there was evidence of uterine and vaginal stimulation (a thorough but fruitless search was made for accessory ovarian tissue). In both these animals, adhesions were present. However, at least in the oldest (No. 22) it is unlikely that ovarian hormones were able to bypass the liver, as blood from the graft (situated in the splenic mesentery) would have to flow in a retrograde manner to reach the

adhesions between the terminal ileum and anterior abdominal wall. Apparently, the liver did not inactivate all the hormones produced.

SUMMARY AND CONCLUSIONS

1. Four of seven viable ovarian grafts placed in the portal circulation of castrate female rabbits surviving 399–673 days were devoid of recognizable follicles or corpora lutea. Two of these were considered definite tumors of the granulosa-cell type.

2. The grafts are described, and their possible pathogenesis and hormone production are discussed.

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Fig. 1.—Tumor from rabbit No. 27. Mag ×480.

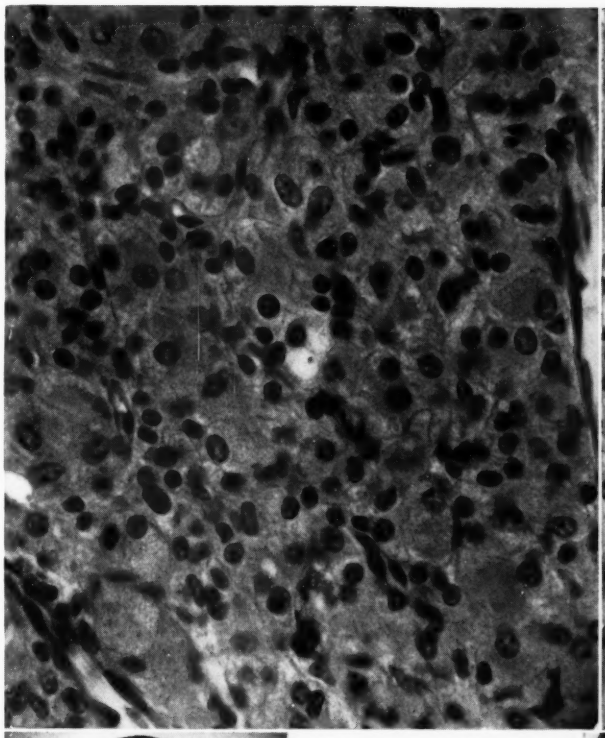
Fig. 2.—Tumor from rabbit No. 22. Mag ×480.

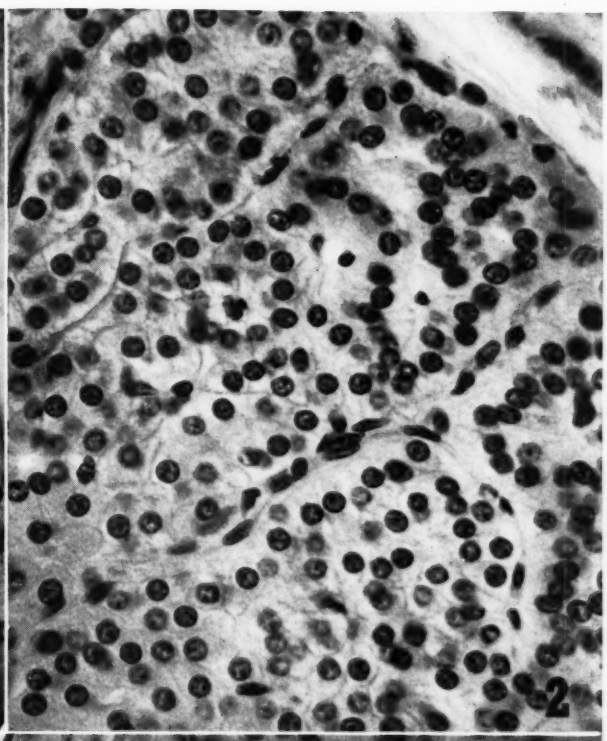
Fig. 3.—Uterine horn from rabbit No. 22 showing estrogenic stimulation. Compare with Fig. 4. Mag. ×5.

Fig. 4.—Uterine horn from untreated rabbit castrated 6 months prior to sacrifice. Compare with Fig. 3. Mag ×5.

Fig. 5.—Transplant from rabbit No. 21. Note apparent infiltration of granulosa cells among the lutein cells of the corpus luteum (on the right). Mag. ×185.

Fig. 6.—Higher magnification of corpus luteum shown in Fig. 5. Note the resemblance between this structure and the tumor in Fig. 1. Mag. ×480.

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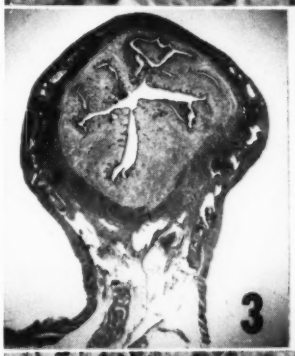
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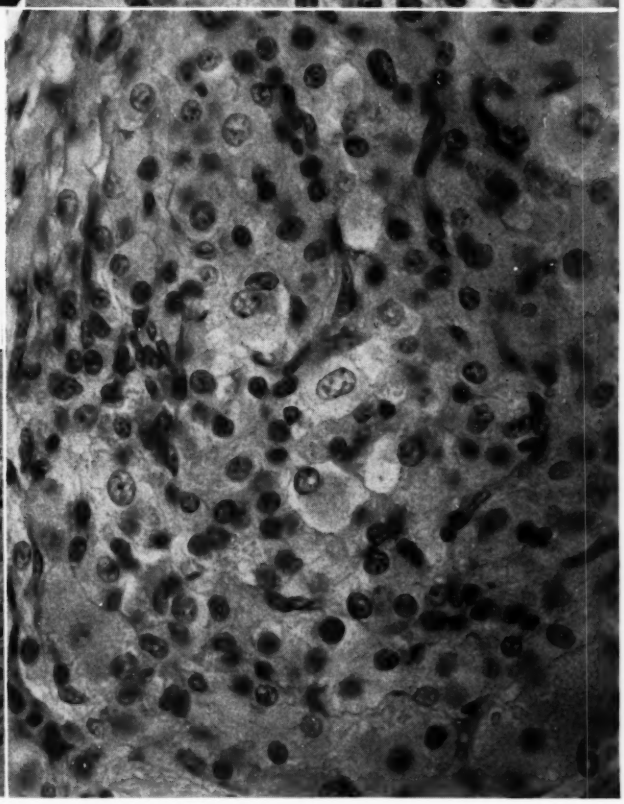

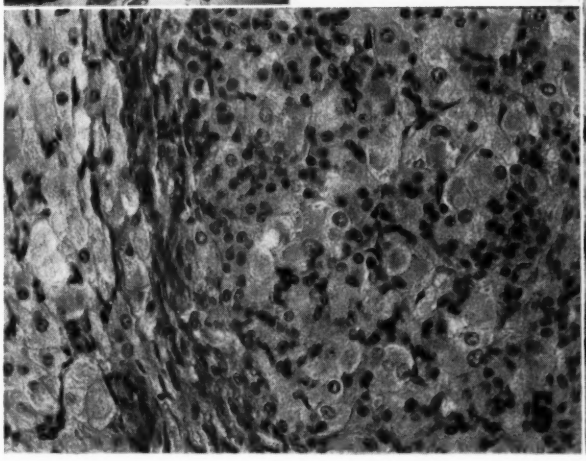
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Carcinogenicity of 1,2-Benzanthracene*

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The carcinogenic activity of 1,2-benzanthracene, tested many times, has usually been reported as negative (3, 6). On the other hand, we recently reported (6) that this compound was relatively potent for the induction of subcutaneous sarcomas in mice of C57 black strain, having a carcinogenic index (4) of 6.4. The difference in results was explained by more adequate testing. This finding appeared important, inasmuch as it required modification of some theories of carcinogenesis, mutagenesis, and growth inhibition which were based on the mistaken belief that this compound was not carcinogenic (6). For this reason, and also because of the basic importance of this compound as a parent substance for other carcinogens, further tests were made, which are here reported. In the meantime, a paper by Greenwood (2) has dealt with some theoretical relations of aromatic bonds to carcinogenic activity in derivatives of 1,2-benzanthracene, and Klein (5) has reported that 1,2-benzanthracene was not additive to 20-methylcholanthrene in skin carcinogenesis in strain DBA mice.

Four additional specimens of 1,2-benzanthracene have now been tested and found to be carcinogenic. The degree of activity has been more precisely titrated in reduced dosage; the results show that this compound is active in small amounts.

METHODS

Four new lots of 1,2-benzanthracene were tested as indicated in Table 1, in addition to the one previously reported. The source of the compounds was as follows: the specimen used in Exp. 1 was provided by Professor Louis F. Fieser; that in Exp. 2 came from L. Light and Co., Ltd.; in Exps. 3 and 4 the specimens were obtained from Eastman Kodak Co. in widely separated years and with different lot numbers (the latter was previously used [6]); in Exps. 5–9, inclusive, the compound was synthesized in this laboratory by methods already known (1). This product was purified by repeated chromatography, and it had the ultraviolet absorption spectrum of 1,2-benzanthracene, as did the other four specimens. Prior to their use in Exps. 2 and 3, these two samples were also purified by chromatography. Subsequent ultraviolet absorption spectrophotometry revealed the presence of no other known carcinogens. The impurities obtained in Exp. 2 were tested and found to have the same order of carcinogenicity as the purified product; the residue in Exp. 3 was not carcinogenic.

The melting points of the impure compounds were higher than the pure, and the higher melting point was again shown by the pure compound on re-test after melting and recrystallization. The melting points in degrees Centrigrade were: In Exp. 1, 160–161; in Exp. 2, 157 (re-melting, 159–160); in Exp. 3, 158–159 (re-melting, 160–161); in Exp. 4, 160–161; in Exps. 5–9, 156–157.

The mice were of both sexes of C57 black strain obtained from the Roscoe B. Jackson Memorial Laboratory or bred in this laboratory from breeding stock obtained from that source. Most of them were between 3 and 4 months old, but a few were older or younger. The injections were made subcutaneously interscapularly, with the desired dose of chemical dissolved or suspended in 0.5 cc. of tricaprylin as solvent. In Exp. 10 this quantity of tricaprylin was injected alone as control. The mice were given laboratory chow pellets and water and housed ten to fifteen in a cage. Weights were taken bi-monthly. Observations were recorded weekly for a month (to detect the early losses of injectate), then monthly until the first tumor appeared, after which occurrence weekly observations were resumed. Mice with tumor were isolated until death. The experiment was terminated in the 28th month, when the mice were about 30 months of age, by autopsying the seventeen survivors. All tumors were finally diagnosed from microscopical sections. The percentage of tumor yield was calculated from the number of sarcomas which occurred in the effective total of mice, i.e. those that survived for 6 months.

RESULTS

All four new specimens of 1,2-benzanthracene, like that previously reported, proved to be sarcogenic. The percentage yields ranged from a high of 55.5 down to 11.4 per cent. The minimum induction time was 161 days, and the average varied from 236 days for the most potent specimen to 438 days for the weakest. The compound was significantly more highly sarcogenic in females than in males ($P > 0.02$). Sixty-four tumors occurred in 216 females that survived 6 months (30 per cent), and 21 sarcomas were found in 115 males (18 per cent). The time of death of mice with induced sarcoma in relation to number of survivors is shown in Table 2. Survival rates were roughly equal in the different experiments.

In an attempt to titrate the minimal tumor dose of 1,2-benzanthracene, the specimen synthesized in this laboratory was injected in progressively decreasing doses, down to 0.05 mg. per mouse (Exps.

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5 to 8). The minimal tumor dose was not revealed, because the smallest amount injected still had a yield of 10.6 per cent. The tumor yield paralleled the reduction in dosage, but the minimal and average induction time were not greatly lengthened by reduced dosage. By extrapolating a straight line graph of percentage yield against the logarithm of the dose, it was calculated from these data that the minimal tumor dose lies between 0.01 and 0.03 mg. When the standard dose of 5 mg/mouse was increased to 10 mg., the tumor yield was not increased (Exp. 9). This is explained by the greater amount of early loss by skin ulceration and extrusion of chemical at this dosage. The tricaprylin controls had no tumors (Exp. 10).

The chief differences between the experiments reported here and those previously described by others are in the greater numbers, longer survival, and use of the subcutaneous test in strain C57 black mice with tricaprylin as solvent (3, 6). A few tests, inadequate by modern standards, have been done with other species of animals. In most of the tests reported with mice, skin painting and relatively short periods of time were used. Subcutaneous injections have been previously made with either no solvent or other solvents and strains of mice in comparatively small numbers for short periods of time. Tricaprylin was used once, but the maximum duration was 14 weeks. 1,2-Benzanthracene was moderately carcinogenic under the conditions of our experiments.

TABLE 1

CARCINOGENICITY OF DIFFERENT SPECIMENS OF 1,2-BENZANTHRACENE IN THE SUBCUTANEOUS TISSUES OF C57 BLACK MICE

Exp. no.	Dose/ mouse (mg.)	No. mice	Effective total mice	No. induced sarcomas	Tumor yield (per cent)	Minimum induction time (days)	Av. induction time (days)	Carcinogenic index
1	5.0	40	34	7	20.6	161	236	8.7
2	5.0	40	35	4	11.4	274	438	2.6
3	5.0	40	37	10	27.0	235	373	7.2
4*	5.0	50	38	8	21.1	161	285	7.4
5	5.0	40	36	20	55.5	183	299	18.5
6	1.0	50	40	15	37.5	152	298	
7	0.2	50	46	11	24.0	183	346	
8	0.05	50	47	5	10.6	242	315	
9	10.0	30	18	5	27.7	306	403	6.9
10	none	40	31	0	0.0			

* Previously reported in reference 6.

TABLE 2

CARCINOGENICITY OF 1,2-BENZANTHRACENE IN C57 BLACK MICE

Exp. no.		Time in months 0	6	9	12	15	18	21	24	27
Sum of 1–5	No. sarcomas	0	1	13	14	15	3	0	3	0
	No. survivors	210	180	145	95	50	39	31	12	8
6	No. sarcomas	0	2	4	5	2	2	0	0	0
	No. survivors	50	40	31	21	14	2	1	1	0
7	No. sarcomas	0	0	1	6	1	1	2	0	0
	No. survivors	50	46	43	32	21	17	7	4	2
8	No. sarcomas	0	0	0	3	2	0	0	0	0
	No. survivors	50	47	43	36	29	20	5	2	2
9	No. sarcomas	0	0	0	2	2	0	1	0	0
	No. survivors	30	18	16	14	11	10	5	3	0
10	No. survivors	50	31	27	24	17	10	8	6	5

SUMMARY

Carcinogenic activity of moderate potency has been demonstrated by five different specimens of 1,2-benzanthracene as tested by sarcoma induction in C57 black mice following subcutaneous injection. The carcinogenic index (Iball) varied from 18.5 down to 2.6. The minimal tumor dose was not determined, because the smallest amount tested (0.05 mg/mouse) was above the tumor threshold dose. Reference is made to the importance of these observations to carcinogens, mutagens, and to other biological phenomena.

ACKNOWLEDGMENT

Gratitude is expressed to Professor Louis F. Fieser for the gift of a specimen of 1,2-benzanthracene and to Dr. Hans Falk, who assisted in the chemical work.

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Studies on Tissue Metabolism by Means of *in Vivo* Metabolic Blocking Technics

I. A Survey of Changes Induced by Malonate in Tissues of Tumor-bearing Rats

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In the years since Warburg found that a high rate of aerobic glycolysis was common in tumors (16), a large series of experiments has been performed in an effort to determine the mechanism of this effect. Under the conditions employed in this laboratory (10, 13, 14), homogenates of a series of transplantable tumors exhibited considerably less oxidative activity than homogenates of liver, kidney, heart, brain, and muscle. Moreover, the oxidation of acetate-1-C^{14} by tumor slices was found to be considerably less than that of normal tissues studied (9); this latter finding has been recently confirmed for a number of different tumors by Weinhouse *et al.* (17). Weinhouse and co-workers have demonstrated that some of the individual enzymes of the Krebs cycle were present in tumors in sufficient concentrations to make oxidations possible (18, 19), but it is clear that the concentrations of several of the oxidative enzymes are lower per gram of tissue in tumors than in the case of tissues such as liver and kidney (10, 19). The lowered capacity of oxidative enzymes relative to glycolytic capacity could account for the high rates of aerobic glycolysis, through the mediation of phosphate systems (10, 14), but there remains the possibility that, in addition to the factor of oxidative *capacity*, there are other factors that operate to yield an imbalance between glycolytic and oxidative *activities*. Since the factors that affect enzyme activity may have as great a significance as the basic enzyme capacities on which they are brought to bear, it seems desirable to attempt to evaluate enzyme activities in the whole animal, since it is apparent that factors governing enzyme activity in the slice and the homogenate are not necessarily operative in the intact animal. One approach to the study of the activity of the Krebs cycle and related reactions *in vivo* was demonstrated by means of fluoroacetate. It was found that little additional citrate was formed by the tumors of the fluoroacetate-treated rats, as compared to very large increases in most normal tissues (11). However, the interpretation of the results was complicated by the necessity for tissues to convert the fluoroacetate to a toxic fluorotricarboxylic acid (2).

Inasmuch as it is possible that fluoroacetate was not effectively converted to the toxic compound by the tumors studied, the use of an inhibitor such as malonate, which did not have to be activated, seemed advantageous. Methods were developed which permitted not only isolation and quantitative determination of succinate, but also isolation and quantitative determination of malonate and a number of other acidic compounds in a single chromatogram; the graphic representation of the titrations in the fractions of these chromatograms has been referred to as the "acid profile" of the tissue (3). Data on the production of succinate after various time intervals and malonate dosages have been presented for several tissues of rats treated with malonate (4).

The present report deals with succinate formation *in vivo* by tumors and normal tissues of rats treated with malonate under the conditions previously established. Associated changes in the acid profiles are also reported.[1]

MATERIALS AND METHODS

Female rats weighing 140–170 gm. and bearing 10-day-old transplantable tumors were used for the study of the acid profiles of normal[2] tissues and the Flexner-Jobling carcinoma, Walker 256 carcinosarcoma, and Jensen sarcoma. These rats were supplied by Dr. G. A. LePage. The aggregate tumor mass,

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[1] A preliminary report on these studies has been presented (5).

[2] In this paper, we refer to the nontumorous tissues of the tumor-bearing rats as "normal" tissues.

obtained from 2 to 4 sites of transplantation, was generally less than 2.5 gm. Mice bearing cutaneous papillomas, induced by a single application of 9,10-dimethyl-1,2-benzanthracene followed by croton oil twice weekly, were furnished by Dr. R. Boutwell, and male rats bearing hepatomas induced by 3'methyl-4-dimethylaminoazobenzene were supplied by Drs. E. C. Miller and J. A. Miller. In the malonate studies, 1.2 ml. of 1 M sodium malonate per 100 grams body weight was injected subcutaneously, and after 1 hour the dose was repeated (4). One hour after the second dose, the animals were decapitated, the blood was collected from the vessels of the neck, and the tissues were excised, frozen in liquid air, and pulverized in the frozen state (6). Each sample contained the tissue from two rats. Skeletal muscle was obtained from the forelegs. The powdered sample was homogenized in a quantity of 0.6 N perchloric acid sufficient to result in a minimal final concentration of 0.3 N perchloric acid. The homogenates were centrifuged, and the supernatant solutions were combined with two wash solutions resulting from resuspension of the protein precipitate in 0.6 N perchloric acid and centrifugation. The combined supernatant solutions were neutralized with 4 N KOH to precipitate the perchlorate ion, and the neutral filtrate was added directly to the anion exchange resin column (Dowex-1, formate form) and permitted to enter the column at the rate induced by gravity. The column was then subjected to a slowly increasing concentration of formic acid, and the acid content of the collected fractions was determined directly by titration with 0.01 N NaOH after the formic acid had been removed by desiccation (3).

Purification of succinic acid.—In liver, kidney, and muscle, the peak containing succinic acid also contained a brown oil resembling caramelized sugar. Sublimation[3] was used as the means for purification of the succinic acid and was effected at 115° C. at 1–2 mm. pressure for 60 minutes in the sublimation apparatus. In the purified samples tested, the purity of the succinic acid was very high, as manifested by a melting point of 188°–189° C. (literature: 189° C.), which was not depressed by the addition of a known sample of succinic acid.

RESULTS

Charts 1 and 2 present representative acid profiles of a number of tissues from control animals, as well as the profiles of tissues from animals treated with malonate. The acid profile of normal kidney has been presented elsewhere (3), along with the variations resulting from treatment of the animals with malonate and fluoroacetate. Inasmuch as there was little difference in the control acid profile of other tumors studied, as compared to the Flexner-Jobling carcinoma, they are not presented, but quantitative data on the contents of their peaks are presented in the tables.

The nature of the acid profiles of tissues from control animals.—Well defined and sharply separated peaks of acidity were found in the acid profiles of all tissues (Charts 1, 2, controls). Large areas of the acid profiles contained no titratable acidity, i.e., less than 0.2 μE per fraction. For the most part, the presence of malonate was associated with the appearance of large, readily recognizable peaks in areas of the profile normally containing little or no titratable acidity (Charts 1, 2, malonate). The acid profiles of control samples of the heart, thymus, lung, spleen, and the Flexner-Jobling carcinoma have three peaks of acidity, numbered 1 to 3 in the graphs, which are qualitatively similar in their positions on the chromatogram (Charts 1, 2). Only two peaks of acidity were consistently noted in the profile of the control sample of blood, and, as was commonly noted, these peaks tended to overlap. A fourth peak was invariably found in the

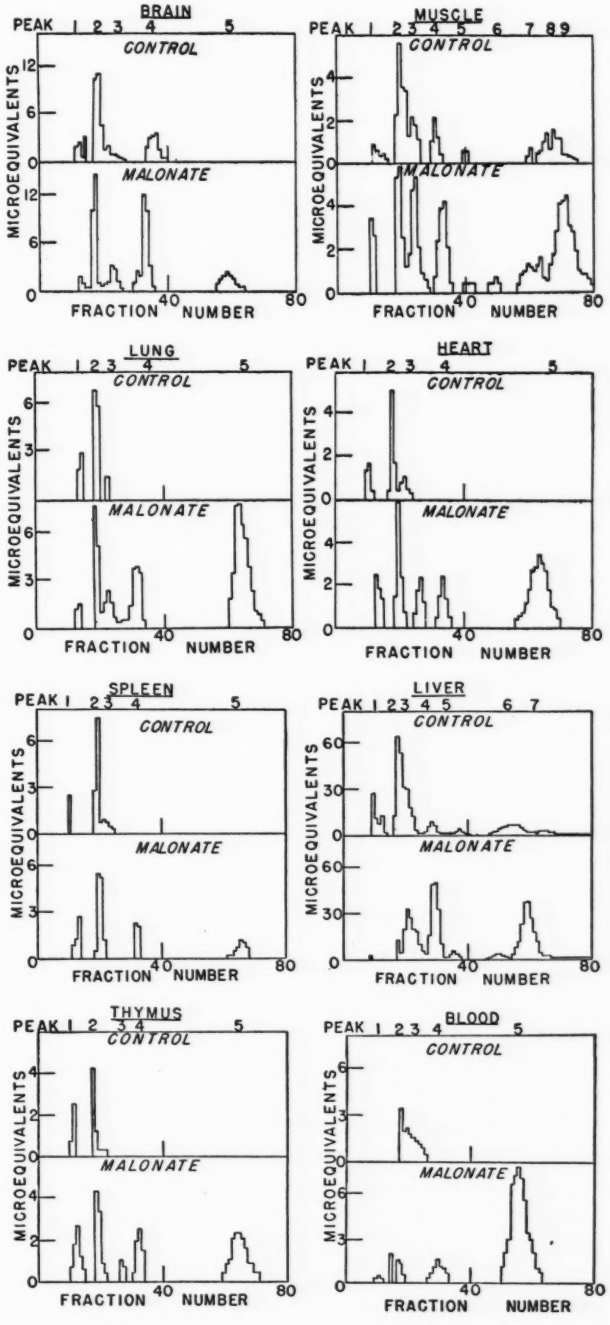

CHART 1.—Representative acid profiles of normal tissues and the effects of malonate on these profiles.

[3] We are grateful to Dr. Charles Heidelberger for suggesting this procedure. The sublimation apparatus was obtained from the Scientific Glass Apparatus Company, Bloomfield, N.J.

acid profiles of control samples of brain (peak 4). This peak may represent a compound unique to the brain, inasmuch as the peak was not equaled quantitatively in other tissues, nor was the residue brownish as it was in the fourth peak of control samples of liver, kidney, and muscle—i.e., the residue in this peak from brain samples was colorless.

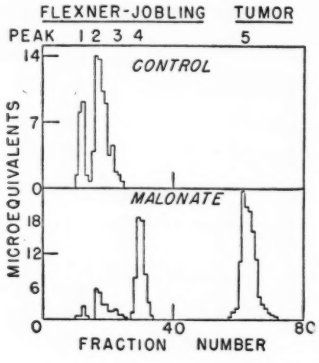

CHART 2.—Representative acid profile of Flexner-Jobling carcinoma and effects of malonate on this profile.

profiles showed changes associated with the presence of malonate, their contents have been studied in greater detail than the contents of other peaks. Peak 1 of thymus and Flexner-Jobling tumor contained a mixture of amino acids as indicated by paper chromatography according to the methods of Benson *et al.* (1). Peak 2 of spleen and the Flexner-Jobling tumor contained the dicarboxylic amino acids and possibly glutathione, as determined by paper chromatography (1) and cation exchange chromatography by the method of Stein and Moore (15).

Acid profiles from tissues of malonate-treated animals.—The chief general effects of malonate treatment were increases in the succinate and malonate peaks. Succinic acid emerged in fractions 28–35 (peak 4 in all cases), and malonic acid emerged in fractions 55–75 (peak 5 in most tissues, peak 7 in the liver, peak 9 in the muscle). In those tissues where little acidity was present in the control profile in fractions 28–35, purity of the succinic acid

TABLE 1

TITRATABLE ACIDITY IN THE SUCCINATE PEAK FOLLOWING THE INJECTION OF MALONATE *in Vivo*

Values are presented in microequivalents per gram wet weight

	CONTROLS			MALONATE INJECTED		
TISSUE	No. samples	Av.	Range	No. samples	Av.	Range
Brain	4	0*		3	2.0	0 – 4.4
Heart	4	0		4	5.0	2.0– 6.6
Lung	6	0		3	5.6	4.0– 7.8
Thymus	4	0		3	9.0	4.0–16.0
Liver	6	2.2	1.2–3.2	5	12.0	9.0–14.4
Kidney	3	0.6	0 –1.0	6	16.4	14.4–18.0
Spleen	4	0		3	12.8	3.8–23.4
Blood	4	0		5	2.6	1.0– 5.4
Muscle	3	1.0	0.6–1.2	5	3.0	1.0– 4.8
Flexner-Jobling carcinoma	8	0		6	9.2	7.8–11.2
Walker 256 carcinosarcoma	2	0		2	8.0	3.0–12.8
Jensen sarcoma	2	0		2	8.8	8.4– 9.0
Hepatoma	2	0		2	6.0	5.4– 6.6
Mouse papilloma	1	0		1	4.4	

* Quantities of less than 0.2μE per gram are assigned the value of zero, in all tables.

Muscle and liver presented the most complex profiles, seven peaks being found in the liver profile and nine peaks in the muscle profile.

Since the acid profile represents the eluate from the acid-soluble fraction of a tissue, the fractions collected must contain not only acids of the Krebs cycle, but also nucleotides, intermediates of glycolysis, certain amino acids, acidic peptides, and other acidic compounds. In general, we have been primarily interested in determining quantitative changes in the peaks as a preliminary to more complete characterization of the constituent compounds. Inasmuch as peaks 1 and 2 of a number of

obtained from these fractions in the malonate profiles was of a high degree, i.e., melting range 178°–183° C. However, in control samples of liver, kidney, and muscle, a brownish residue was left after desiccation in the tubes comprising peak 4 (Chart 1, Table 1). This residue also contaminated the crystals of succinic acid found in this peak from samples prepared from the corresponding tissues of malonate-treated animals. Purified succinic acid was obtained in these cases by sublimation (see above). The greatest consistent increases in the titratable acidity of the succinate peak were found in the liver and kidney, with thymus and spleen

also forming significant quantities. All tumors studied also accumulated large amounts of succinate in the presence of malonate (Table 1). Little succinate accumulated in the brain, muscle, and blood. Some succinate was found in the blood (4) when very high levels of of malonate were present.

Table 2 presents the content of titratable acidity in the malonate peak for individual tissues. The high levels of malonate in the blood and kidney attest to their respective roles in the transportation and excretion of the antimetabolite (4). The relatively low level of malonate in the brain may account for the low level of succinate formed. In general, a fairly uniform relationship was found for the malonate and succinate content of a given tissue (4), but there was a wide variation from tissue to tissue.

TABLE 2

TITRATABLE ACIDITY IN THE MALONATE PEAK FOLLOWING INJECTION OF MALONATE *in Vivo*

Values are presented in microequivalents per gram wet weight of tissue. The number of samples corresponds to that given in Table 1.

	Controls		Malonate injected	
Tissue	Av.	Range	Av.	Range
Brain	0		2.8	2.0– 3.4
Heart	0		18.4	9.8–26.0
Lung	0		16.0	9.0–27.4
Thymus	0		21.0	2.2–31.6
Liver	2.4	1.4–4.2	14.2	13.2–16.4
Kidney	0.3	0.0–0.6	44.0	21.0–75.0
Spleen	0		12.8	7.4–23.4
Blood	0		24.6	13.6–41.2
Muscle	2.0	1.2–3.2	8.8	6.4–11.0
Flexner-Jobling carcinoma	0		17.6	10.2–22.2
Walker 256 carcinosarcoma	0		5.2	4.2– 6.0
Jensen sarcoma	0		8.0	7.0– 8.8
Hepatoma	0		12.0	7.8–16.0
Mouse papilloma	0		4.4	

In addition to changes in the succinate and malonate peaks, other peaks, not yet completely characterized, also showed significant changes. Increases in the titratable acidity of peak 1, comprising fractions 9–14, were noted in the thymus, spleen, and mouse papilloma (Table 3) of the malonate-treated animals. Notable decreases in this peak were observed in the samples of Flexner-Jobling carcinoma (Chart 2) and brain of malonate-treated rats. The presence of malonate was associated with marked increases in titration in peak 2 of the spleen and thymus (Table 4) and marked decreases of the content of this peak in the Flexner-Jobling tumor. Although little change was noted quantitatively in the total titratable acidity of the peak in other tissues, the possibility of variation in the concentration of components within the peak cannot be ruled out.

It is apparent from the acid profile of the Flexner-Jobling tumor that the presence of malonate is associated with a decrease in the first two peaks as well as an increase in the succinate peak (Chart 2). This inverse relationship between the titratable acidity of the succinate peak and the total titratable acidities of the first two peaks in this tumor was found to hold true over a wide range of concentrations of malonate (Chart 3) in the tumor. The amino acid content of peaks 1 and 2 was determined by the ninhydrin method of Moore and Stein (8), and the decrease in amino acid content was proportional to the diminution in titration. The contents of peak 2 from a control tumor were chromatographed on cation exchange columns (15); 44 per cent of the content of the peak was glutamate, and 29 per cent was aspartate. The re-

TABLE 3

EFFECT OF MALONATE ON THE CONTENT OF PEAK 1

Values are microequivalents per gram wet weight of tissue. The number of samples corresponds to that given in Table 1.

	Controls		Malonate injected	
Tissue	Av.	Range	Av.	Range
Brain	2.2	1.2– 3.6	0.3	0.0– 0.9
Heart	4.0	2.1– 5.8	4.0	2.4– 5.9
Lung	2.6	2.1– 3.3	2.5	1.4– 3.4
Thymus	9.0	7.9–10.8	17.9	12.4–23.0
Liver	3.0	0.8– 4.8	1.6	1.1– 1.9
Kidney	4.6	3.9– 6.0	4.8	2.8– 7.5
Spleen	3.4	2.9– 3.7	12.1	7.9–16.7
Blood	0.6	0.4– 0.7	0.6	0.5– 0.9
Flexner-Jobling carcinoma	2.3	1.7– 3.6	0.4	0.0– 0.9
Walker 256 carcinosarcoma	2.3		2.8	2.5– 3.0
Jensen sarcoma	2.4	2.2– 2.6	1.3	0.0– 2.6
Hepatoma	1.9	0.9– 2.9	4.4	2.5– 6.3
Mouse papilloma	3.0		8.0	

TABLE 4

EFFECT OF MALONATE ON THE CONTENT OF PEAK 2

Values are microequivalents per gram wet weight of tissue. The number of samples corresponds to that given in Table 1.

	Controls		Malonate injected	
Tissue	Av.	Range	Av.	Range
Brain	9.3	6.7–11.2	8.7	6.7–10.4
Heart	7.6	7.2– 8.1	7.8	6.6– 9.1
Lung	6.1	5.0– 8.4	6.9	3.2– 8.7
Thymus	4.1	2.0– 8.0	23.4	10.0–30.6
Liver	13.8	7.2–20.6	13.0	10.6–14.7
Kidney	10.8	8.8–12.9	7.7	5.3– 8.5
Spleen	9.4	8.0–12.2	21.2	17.2–26.3
Blood	2.8	1.9– 4.9	2.6	1.5– 3.7
Flexner-Jobling carcinoma	8.3	6.0–12.1	1.8	0.4– 4.2
Walker 256 carcinosarcoma	4.5	3.4– 5.5	3.7	
Jensen sarcoma	7.5	6.5– 8.5	2.5	
Hepatoma	4.5	4.2– 4.8	5.7	4.7– 6.7
Mouse papilloma	7.2		6.7	

maining 27 per cent of the titratable acidity of the peak emerged considerably earlier than either of the acidic amino acids, which are among the first of the known amino acids to emerge from the column in this type of chromatographic procedure. In the same peak from the tumor of a rat treated with malonate, less than 25 per cent of the markedly diminished content of the peak was found in the glutamate and aspartate fractions from the cation exchange columns.

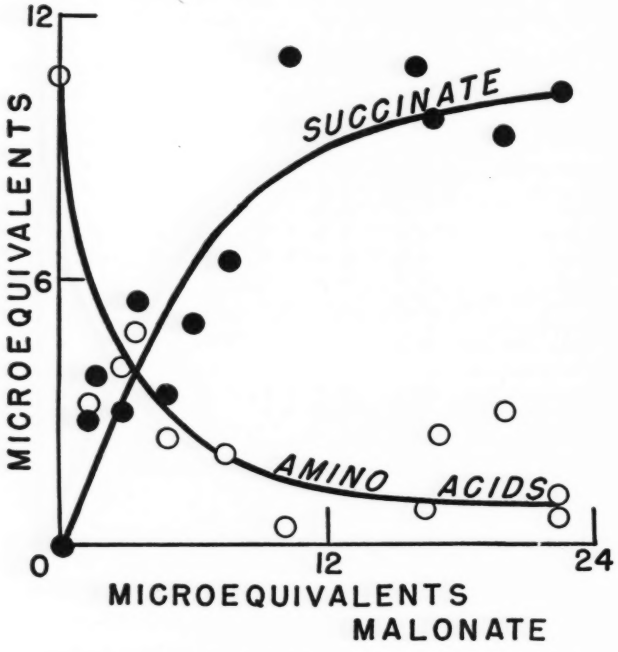

CHART 3.—The effect of increasing malonate concentration on succinate content and combined content of the amino acid peaks in the Flexner-Jobling tumor. All figures are in microequivalents per gram wet weight of tumor. Each point represents the average of 1–3 determinations, except for the control which represents the average of eight determinations.

A possible explanation for the failure of other tumors (Tables 1, 3, 4) to exhibit the inverse relationship between the early peaks containing amino acids and the succinate peak may be the degree of necrosis present even in fairly small tumors. In fifteen studies with Flexner-Jobling tumors, averaging 600 mg/tumor mass (each sample contained eight tumor masses pooled from two rats), this inverse relationship was consistently found. When larger, visibly necrotic Flexner-Jobling tumors were tested, these relationships between early peaks of the acid profile and the succinate peak were not clearly demonstrable. To test this point in the Jensen tumor, two groups of rats were injected with malonate, one group bearing larger tumors, visibly necrotic after dissection, and the other group bearing small tumors from which any suspect material was excised. About 20 per cent less succinate accumulated in the necrotic tumors in terms of microequivalents per gram, but in the profile of these tumors peak 2 showed a slight increase, while in the profile of the non-necrotic tumor the content of peak 2 dropped from 7.5 μE per gram to 2.5 μE per gram.

DISCUSSION

The succinate accumulated by a given tissue depends on the completeness of the malonate block, diffusion of succinate to and from the blood, and the activity of endogenous formative pathways. The *completeness of the malonate block* is dependent upon the total inhibitor present in the tissue, the succinate concentration within the tissue, the permeability of the cells and the permeability of the mitochondria, as well as other factors yet undefined. The extent of *diffusion of succinate* to and from the blood is not known, but, in most instances, the succinate content of the blood was very low relative to the amounts found in the tissues (4). The *formative pathways* to succinate funnel through the Krebs cycle, the intermediates of which are normally present only in trace quantities. Accordingly, outside sources for intermediates of the cycle are probably utilized in the accumulation of large concentrations of succinate. Thus, α-ketoglutarate may arise from oxidative deamination of glutamate, while oxalacetate may be formed from CO_2 fixation or oxidative deamination of aspartate. These acids can then proceed through the intermediate steps of the Krebs cycle to succinate. If the levels of amino acids within tissues are reflected by peaks 1 and 2 of the acid profiles, it is evident that the processes maintaining the levels of amino acids in the tissues (acting via peptidases or proteinases) are very active and sensitive or that CO_2 fixation is an active process, for in most instances little change in these peaks was found. Moreover, in thymus and spleen, marked *increases* in the contents of these peaks were noted. In contrast, in the Flexner-Jobling tumor, these first two peaks were virtually eliminated, indicating that replacement of amino acids shifting into the succinate pool from the endogenous proteins was limited. The slight increase in the level of the plateau of succinate accumulation in this tumor (Chart 3) may indicate utilization of precursors such as free glutamate from exogenous sources, after the endogenous sources are depleted. If succinate content were the only data available in these experiments, one might conclude that the oxidative functions of the Krebs cycle and related reactions of tumors *in vivo* were quantitatively similar to those of normal tissues. It is evident that, in the Flexner-Jobling tumor, much of the succi-

nate may have come from free glutamate and related compounds and circumvented the condensing enzyme, aconitase, and isocitric dehydrogenase. It is possible that this active pathway to succinate may be used for equilibration of amino acids within the tumor to fit the protein pattern required for growth (7).

The accumulation of succinate in the tumor makes possible an approach to the problem of oxidative activity *in vivo* by means of isotopes, and it has been found that the radioactivity of injected acetate-1-C^{14} was rapidly incorporated into the succinate of a variety of normal tissues, while in the Flexner-Jobling carcinoma the extent of incorporation into succinate was very slight (5, 12).

SUMMARY

1. The acid profiles for a number of tissues of tumor-bearing rats indicate the variability of the acidic components from tissue to tissue. Two peaks of acidity were found in control samples of the blood, three in the heart, lung, thymus, spleen, and tumors, four in the brain, seven in the liver and nine in the muscle.

2. In animals treated with malonate, succinate accumulated in tumors to an extent comparable to the other tissues, i.e., 3–5 μM per gram wet weight in 2 hours.

3. In most tissues of malonate-treated rats, the content of the peaks containing amino acids either remained constant or increased; in the Flexner-Jobling tumor, a marked decrease was noted in titration, amino acid content, and content of glutamate and aspartate, corresponding to the increase in succinate.

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The Incorporation of Formate-C^{14}, Glycine-2-C^{14}, Adenine-4,6-C^{14}, and Phosphate-P^{32} into Nucleic Acids

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In recent years numerous studies have been made on the relative turnover rates of desoxypentosenucleic acid (DNA) and pentosenucleic acids (PNA), employing as labeling precursors such different tracers as sodium phosphate labeled with P^{32}, formate labeled with C^{14}, and glycine and adenine labeled either with C^{14} or N^{15}. Considerable controversy has developed as to which one of these tracer compounds gives an accurate measurement of the "relative turnover-rate" of the nucleic acids.

In order to determine the "absolute turnover-rate" of the nucleic acids, it is necessary to know the specific activity of the immediate precursor of the nucleic acids. Since even the identity of this immediate precursor is not known at present, it is necessary to find another way to compare the rate of incorporation of the following precursors—inorganic phosphate (P^{32}), formate-C^{14}, glycine-2-C^{14}, and adenine-4,6-C^{14}—into the nucleic acids. Judging from the varied metabolic paths of these labeling agents, their "absolute availability" to the nucleic acids is probably not the same. Assuming that the labeling agent within any one tissue is equally available to both PNA and DNA, it seems advisable to compare the specific activity (per cent incorporation of radioactivity/mg nucleic acid phosphorus) of PNA to that of DNA, with the use of a common precursor. If this specific activity ratio is constant irrespective of the precursor used, then the molecules of PNA and DNA are renewed *in toto*, or at least the labeled part of the molecule is renewed at an equal relative rate in PNA and DNA.

In several earlier papers the PNA:DNA specific activity ratios were compared. These ratios as reported in the literature vary considerably, not only for the different precursors employed but also from author to author utilizing the same precursor. The findings for the PNA:DNA ratios in rat livers are tabulated in Table 1.

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The inconsistency of the data presented in Table 1 may possibly be due to the lack of duplication of the experimental procedure of one author by the procedure of another. The time interval elapsing between the administration of the labeling agent and the sacrifice of the animal varies among the different experiments. Since the incorporation of the active precursors into the following three nucleic acids—cytoplasmic PNA (cPNA), nuclear PNA (nPNA), and DNA—does not proceed at the same rate, the ratios of the specific activities of these nucleic acids at various time intervals are not the same. Other variable factors which must be considered are the mode of administration, the quantity of the labeling agent, and the degree of utilization of the labeling agent by the different nucleic acids. In qualification of the last mentioned factor, it has been suggested by Reichard (18) that adenine cannot be utilized in the synthesis of the purines of DNA.

Hoping to clarify the inconsistency of the findings of the various investigators, we have attempted to compare the specific activities of the nucleic acids using sodium phosphate (P^{32}), formate-C^{14}, glycine-2-C^{14}, and adenine-4,6-C^{14} as precursors under identical experimental conditions.

EXPERIMENTAL

Adult male A strain mice weighing between 20 and 25 gm. were used for all the experiments. The mice were fasted 24 hours prior to the experiment. They received intraperitoneal injections of the labeled precursor about 9 A.M. and were sacrificed with ether precisely 4 hours later, except in the one 24-hour experiment presented in Tables 3 and 4. The livers, intestines, and spleens were removed, and the isolation of nucleic acids begun as soon as possible. The tissues from the following number of mice were combined for the isolation: twelve livers (except where otherwise indicated), two intestines, and four spleens. The procedure for the isolation of the liver nucleic acids (17) and the DNA from the spleen and intestine (14) has been presented previously by the authors. The method used for the experiments on carbon labeling was identical to the one described for the phosphorus-labeled nucleic acids, except that it was found necessary in the case of the carbon-labeled PNA

to reprecipitate the nucleic acid 5 times further at the end of the isolation procedure in order to attain a constant specific activity on additional reprecipitation. The determination of specific activity of the phosphorus-labeled nucleic acid has been described in two earlier papers (14, 17). The specific activity of the nucleic acid is expressed as counts/minute/mg of nucleic acid phosphorus divided by counts injected/mouse normalized for the weight of the mouse.

All the precursors injected were in isotonic saline. The procedure used for the 24-hour experiment differed in some detail from the other experiments. The mice were fasted only during the duration of the experiment. Since adenine is known to have toxic properties, controls for the labeled adenine-injected animals simultaneously received inactive adenine and either P^{32} or formate-C^{14}.

RESULTS AND DISCUSSION

It is apparent from Table 2 that the relative uptake of P^{32}, formate-C^{14}, glycine-2-C^{14}, and adenine-4,6-C^{14} into liver DNA is remarkably similar in terms of percentage of precursor incorporated per milligram of nucleic acid. This suggests that the various precursors of substances that metabolically precede synthesis of DNA may have approximately the same magnitude and turnover.

In the 24-hour experiment we compared the incorporation of adenine-C^{14}, formate-C^{14}, and P^{32}. The results are tabulated in Tables 3 and 4.

TABLE 1

RATIOS OF SPECIFIC ACTIVITIES OF PNA/DNA IN RAT LIVER UTILIZING VARIOUS PRECURSORS

Precursor	Mode of administration	Time after administration of precursor	Ratio of specific activity of PNA/DNA	Author
$Na_2HP^{32}O_4$	subcutaneous	2 hours	33	Hammersten and Hevesy (13)
"	intraperitoneal	24 hours	40*†	Barnum and Huseby (2)
"	"	"	100‡	Barnum and Huseby (2)
"	parenterally	3 days	5	Brues, Tracy, and Cohn (6)
Glycine-2-C^{14}	by stomach tube	24 hours	1.2	Le Page and Heidelberger (17)
Glycine-2-C^{14}	"	24 hours	2.2†	Heidelberger and Le Page (14)
Glycine-N^{15}	subcutaneous	6 hours	4.1	Bergstrand, Eliason, Hammarsten, Norberg, Reichard, and Ubisch (3)
Glycine-N^{15}	fed	5 days	2.5–3	Furst and Brown (10)
Adenine-8-C^{14}	fed	5 days	50– 60	Furst and Brown (10)
Adenine-1,3-N^{15}	fed	5 days	73–100	Furst, Roll, and Brown (11)

* Mouse liver.

† Our calculations taken from their data of the specific activity of the PNA and DNA purines.

‡ Nuclear PNA/DNA ratio.

TABLE 2

AVERAGES OF SPECIFIC ACTIVITY × 10^4 OF DNA LABELED WITH $Na_2HP^{32}O_4$, FORMATE-C^{14}, GLYCINE-2-C^{14}, AND ADENINE-4,6-C^{14} IN LIVER

Labeling agent	DNA*
$Na_2HP^{32}O_4$	1.26±0.14
Formate-C^{14}	2.74±0.24
Glycine-2-C^{14}	2.20±0.29
Adenine-4,6-C^{14}	3.28±0.36

* Incorporation of precursor was measured at 4 hours.

TABLE 4

COMPARISON OF SPECIFIC ACTIVITY RATIOS IN LIVER

(24-hour experiment)

cPNA:DNA	Probability*
Adenine-4,6-C^{14} vs. $Na_2HP^{32}O_4$	*0.18*†
Adenine-4,6-C^{14} vs. $Na_2HP^{32}O_4$+inactive adenine	*0.46*
Formate-C^{14} vs. $Na_2HP^{32}O_4$	0.034
Formate-C^{14} vs. formate-C^{14}+inactive adenine	0.05
Formate-C^{14} vs. $Na_2HP^{32}O_4$	0.01

* Probability found from the distribution of t (8).

† Nos. in italics represent no significant difference.

$$t = \frac{M_1 - M_2}{\sqrt{(\sigma M_1)^2 \pm (\sigma M_2)^2}}$$ where M = mean.

σM = standard error of mean.

TABLE 3

SPECIFIC ACTIVITY × 10^4 OF cPNA AND DNA IN LIVER UTILIZING ADENINE-4,6-C^{14}, FORMATE-C^{14}, AND $Na_2HP^{32}O_4$ AS PRECURSORS

(24-hour experiment)

Precursors	cPNA	DNA	cPNA:DNA
Adenine-4,6-C^{14}*	127 ±4.7	5.13±0.8	24.8 ±4.0
Formate-C^{14}	9.3 ±1.7	1.82±0.22†	5.11±1.1
Adenine‡+Formate-C^{14}	1.096±0.017	0.93±0.075	2.1 ±0.16
$Na_2HP^{32}O_4$	44.4 ±1.7	2.72±0.54	16.3 ±3.1
Adenine‡+$Na_2HP^{32}O_4$	38.6 ±0.62	1.86±0.26	20.8 ±2.95

* Specific activity = 4.2 μc/mg. Injected 0.95 mg = 7.03 μM/mouse.

† We have no explanation why this value is lower than the value for the 4-hour experiment; however, it represents only 8 mice and may be in error.

‡ Inactive adenine injected = 0.95 mg. = 7.03 μM/mouse.

Concerning the cPNA:DNA ratio, the results are similar to those of the 4-hour experiments. The cPNA:DNA ratio of the formate-labeled nucleic acid is much lower than the same ratio of either the adenine- or the phosphorus-labeled nucleic acids. The cPNA:DNA ratio obtained with adenine is the same as that with P^{32}, within experimental error.[1]

The specific activities obtained with the four precursors in the 4-hour experiments are shown in Tables 5, 6, 7, and 8. As seen in Table 9, the ratios of specific activity of cPNA to DNA with formate and glycine as precursors are considerably lower than the same ratio for P^{32}. However, the specific activity ratio for nPNA and cPNA with formate is comparable to that with phosphate. This lower ratio of PNA:DNA, when glycine and formate were used as precursors as compared to inorganic phosphate, possibly indicates an incorporation of phosphate independent of total nucleotide renewal. However, the findings of several authors may give an explanation as to why there could be a lower ratio of PNA:DNA with glycine and formate as compared to phosphate, and yet no "independent exchange" of the phosphate moiety without the C–N skeleton of the purines being affected. Elwyn and Sprinson (7) and Totter, Volkin, and Carter (19) showed that both glycine and formate are incorporated into the pyrimidine, thymine, but not into the pyrimidines,

TABLE 5

SPECIFIC ACTIVITY × 10^4 OF NUCLEIC ACIDS LABELED WITH $Na_2HP^{32}O_4$*

(4-hour experiment)

Tissue	DNA	cPNA	nPNA	No. mice
Liver	0.92±0.24	19.25±0.3	105.5±4.2	24
	1.10±0.12	12.8 ±2.1		12†
	1.82±0.26	24.9 ±1.8		16†
	1.17±0.16	17.2 ±1.1	63.5±5.1	36
	1.61‡	15.8 ±0.52	54.2±2	24
Av.	1.26±0.14	17.9 ±1.3	71.4±8.5	
Intestine	22.8 ±0.9			24
	15.3 ±0.9			12
	38.6 ±2.4			16
	32.5 ±1.0			36
	22.1 ±1.5			24
Av.	26.7 ±1.2			
Spleen	53.3 ±4.6			24
	37.2 ±4.2			12
	55.3 ±3.6			16
	47.6 ±3.0			36
	43.1 ±2.7			24
Av.	48.2 ±1.7			

* Amount of $Na_2HP^{32}O_4$ injected = 0.05 μM/mouse.
† Two livers pooled for DNA and cPNA determinations.
‡ This value represents only twelve mice.

TABLE 6

SPECIFIC ACTIVITY × 10^4 OF NUCLEIC ACIDS LABELED WITH FORMATE-C^{14}*†

(4-hour experiment)

Tissue	DNA	cPNA	nPNA	No. mice
Liver	3.10±0.36	7.70±0.52	29.4±3.7	48
	2.90±0.52	4.60±0.17		12‡
	2.20±0.6	9.58±0.72	25.1±3.7	36
Av.	2.74±0.24	7.99±0.45	27.6±2.4	
Intestine	50.6 ±2.7			48
	70.4 ±6.1			12
	69.0 ±3.5			36
Av.	60 ±2.6			
Spleen	63.4 ±3.8			48
	69.1 ±1.8			12
	55.6 ±1.9			36
Av.	61.1 ±3.1			

* Specific activity of formate-C^{14} = 19.12 μc/mg.
† Amount of formate injected = 22.8 μM/mouse.
‡ Two livers pooled for DNA and cPNA determinations.

TABLE 7

SPECIFIC ACTIVITY × 10^4 OF NUCLEIC ACIDS LABELED WITH ADENINE-4,6-C^{14}*†

(4-hour experiment)

Tissue	cPNA	DNA	No. mice
Liver	49.0±1.4	3.28± 0.36	16
Intestine		126±12.0	16
Spleen		69±11.0	16

* Specific activity of adenine = 4.2 μc/mg.
† Amount of adenine injected = 12.4 μM/mouse.

TABLE 8

SPECIFIC ACTIVITY × 10^4 OF NUCLEIC ACIDS LABELED WITH GLYCINE-2-C^{14}*

(4-hour experiment)

Tissue	cPNA	DNA	No. mice
Liver	6.36±0.75	1.82±0.22	10*†
	7.66±0.37	2.37±0.38	22‡§
Av.	7.25±0.36	2.20±0.29	
Intestine		21.2 ±1.6	10
		20.7 ±1.9	22
Av.		20.9 ±1.5	
Spleen		24.3 ±2.1	10
		16.9 ±0.62	22
Av.		19.2 ±1.5	

* Specific activity of glycine = 11.1 μc/mg.
† Amount of glycine injected = 24 μM/mouse.
‡ Specific activity of glycine = 12 μc/mg.
§ Amount of glycine injected = 7.8 μM/mouse.

and yet no "independent exchange" of the phos-

Totter, Volkin, and Carter (19) showed that both glycine and formate are incorporated into the pyrimidine, thymine, but not into the pyrimidines,

[1] It is interesting to note (Tables 3 and 4) that the availability of exogenous adenine diminishes the incorporation of formate into the purines of both DNA and PNA. This same effect has been noted by Goldthwait and Bendich (11). Since the simultaneous injection of adenine with phosphorus has no marked effect on the incorporation of phosphorus into the nucleic acid molecule, the effect of adenine is not on the rate of synthesis of nucleic acid but is apparently an inhibition of formate incorporation when exogenous adenine is available.

uracil or cytosine. This would mean that there are two bases labeled in PNA, while in DNA three bases are labeled. An additional explanation is suggested by the fact that Löw (16) found that the C^{14} of glycine-1-C^{14} is incorporated into the pentoses of PNA. Bernstein (4) has found that the C^{14} of formate is incorporated into ribose. Glycine-2-C^{14} may be incorporated into the pentoses of the nucleic acids, but as yet this has not been demonstrated. Our findings that the "formate nPNA: cPNA" ratio is not significantly different from the same ratio with P^{32} suggests that the difference in the DNA:PNA ratio may be due to a difference in the labeling of the two sugars. If either the amount of glycine than formate, whereas the amounts in spleen and intestine are approximately equal. This means that the availability of glycine for the formation of nucleic acid in the intestine and spleen may be considerably less than the availability of formate in these tissues.

With the use of adenine-C^{14}, which is incorporated into nucleic acids both as adenine and as guanine (5), and in comparing the liver cPNA:DNA ratio with that of the P^{32} ratio, it is seen (Tables 9 and 10) that they are the same within experimental error. This equality does not agree with results obtained by Furst, Roll, and Brown (10) employing adenine as the labeling agent in rats. Furst *et al.* find a

TABLE 9

SPECIFIC ACTIVITY RATIOS OF THE VARIOUS NUCLEIC ACIDS

(4-hour experiments)

	Formate-C^{14}	Glycine-2-C^{14}	Adenine-4,6-C^{14}	$Na_2HP^{32}O_4$
Liver nPNA: cPNA	3.45 ±0.36			3.99 ±0.55
Liver nPNA: DNA	10 ±1.24			56.7 ±9.2
Liver cPNA: DNA	3.0 ±0.31	3.23 ± .42	15.0 ±1.7	14.2 ±1.86
Intestine DNA: liver DNA	22 ±2.16	9.5 ±1.4	30.3 ±4.4	21.2 ±2.54
Spleen DNA: liver DNA	22 ±2.0	8.7 ±1.3	16.6 ±3.1	38.3 ±4.4

TABLE 10

COMPARISON OF THE SPECIFIC ACTIVITY RATIOS OF THE VARIOUS NUCLEIC ACIDS

(4-hour experiments)

	PROBABILITY P			
	Formate-C^{14} vs. $Na_2HP^{32}O_4$	Glycine-2-C^{14} vs. $Na_2HP^{32}O_4$	Adenine-4,6-C^{14} vs. $Na_2HP^{32}O_4$	Glycine-2-C^{14} vs. formate-C^{14}
Liver nPNA: cPNA	*0.44*			
Liver nPNA: DNA	0.001			
Liver cPNA: DNA	0.001	0.001	*1.00*	*0.22*
Intestine DNA: liver DNA	*1.00*	0.001	*0.12*	0.001
Spleen DNA: liver DNA	0.01	0.001	0.01	0.001

Nos. in italics represent no significant difference.

amount of radioactive carbon or the rate at which it is incorporated is greater in the desoxypentose than in the pentose sugar, the ratio of PNA:DNA would be lower in the case of the nucleic acid labeled with formate and glycine than with P^{32}. To clarify these points, the authors are planning to do a similar series of experiments with chromatographically purified compounds.

In another attempt to compare the different precursors, the specific activity ratios of spleen DNA:liver DNA and intestine DNA:liver DNA obtained with different precursors were measured. As the data in Tables 9 and 10 show, the intestine DNA:liver DNA ratio does not differ significantly in the case of adenine, formate, and phosphorus, while the glycine ratio is lower. The spleen DNA:liver DNA ratio is the same within experimental error only for formate and adenine. This may be explained by our preliminary finding that the liver takes up a proportionately larger ratio of total liver PNA:DNA of 73:1. Brown (5) attributes this "either to the fact that adenine does not serve as a precursor of the DNA purines, or to the fact that the DNA purines are not in a rapid dynamic equilibrium." In a later paper, Furst and Brown (9) compare the incorporation of N^{15}-labeled glycine and C^{14}-labeled adenine into liver nucleic acids by the simultaneous administration of the two labeling agents. They find a total liver PNA:DNA ratio of 50–60:1 with the use of adenine and 2.5–3:1 with the use of glycine. They interpret these results to mean that there exist two mechanisms of DNA synthesis. Reichard (18) believes that the "difference in the turnover ratio DNA to PNA between adenine and phosphorus might indicate that some portion of the phosphate moieties of nucleic acid may be exchanged without the purines being affected." On the other hand Abrams and Goldinger (1), employing adenine-8-C^{14} and guanine-8-C^{14} in a study

on the purine renewal rates in bone marrow preparations, find the same renewal rates as Stevens[2] in an identical experiment employing P^{32} as inorganic phosphate. Abrams and Goldinger suggest that "there can be no independent turnover of purines from an intact polynucleotide structure, but rather that entire nucleotides must be incorporated as units." Our data with adenine and phosphate are in agreement with Abrams and Goldinger's suggestion. The close agreement between adenine-C^{14}- and P^{32}-labeled nucleic acids both in the 4-hour and the 24-hour experiments is an indication that, once synthesis of the nucleic acid molecule is complete, there is no independent renewal of the carbon or phosphorus moiety.

SUMMARY

The incorporation of formate-C^{14}, glycine-2-C^{14}, adenine-4,6-C^{14}, and inorganic phosphate (P^{32}) into liver nucleic acids, splenic DNA, and intestinal DNA has been measured in adult male A strain mice under identical experimental conditions. The liver cPNA:DNA, nPNA:cPNA, spleen DNA:liver DNA, and intestine DNA:liver DNA specific activity ratios observed with the four labeling agents have been compared. From the observed results it is concluded that there is not an independent turnover of any one fraction of the nucleic acid molecule.

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[2] Quotation of Stevens by Abrams and Goldinger (1).

Effects of Atabrine and of Certain Related Substances on the Development of Liver Tumors Due to Azo Dyes*

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There are several reasons for considering the possible effect of atabrine (quinacrine) upon the development of liver tumors due to azo dyes. Hellerman *et al.* (17) have shown that atabrine as well as quinine, auramine, and certain other antimalarial substances inhibit the action of riboflavin-containing enzymes *in vitro*. The development of liver tumors due to certain of the azo dyes is sensitive to the level of riboflavin in the diet (14, 20, 30), and riboflavin-containing enzymes are involved in the detoxification and degradation of the azo dyes *in vitro* (27). Any substance decreasing the effectiveness of riboflavin in the liver might, therefore, be expected to enhance the carcinogenicity of 4-dimethylaminoazobenzene (DAB). Another possible connection between atabrine and liver tumors is the fact that the drug apparently damages the liver of certain experimental animals. Both microscopic and gross liver changes have been reported, including pigmented cells, irreversible necrotic lesions, and impaired responses to the bromsulphalein test (5, 34, 38).

In the present study quinine, atabrine, and auramine were fed to rats simultaneously with the carcinogen DAB or alternately (7) with the more potent carcinogen, 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB). As a quantitative measure of the additive effect of atabrine under the latter conditions, other carcinogens were also fed during the middle period between two dosages of 3′-Me-DAB. For some of the groups, the storage and excretion of atabrine were measured and the effect on the riboflavin content of the livers determined.

METHODS

Groups of fifteen young adult male or female rats, weighing approximately 200 gm., were housed in wire-bottomed cages and fed diet I (11, 30), which consisted of: extracted casein, 12; salts, 4; corn oil, 5; and glucose monohydrate, to 100, with vitamins added at the following levels in mg/kg diet: thiamine, 3; pyridoxine, 2.5; calcium pantothenate, 7.5; and riboflavin, 1. In the initial series, choline chloride was added at 30 mg/kg of diet; thereafter, the diets contained 1 gm choline/kg. Each rat received 2 drops of halibut liver oil every 4 weeks. 4-Dimethylaminoazobenzene (DAB) was incorporated into the diets at 0.06 per cent, and the diet was fed continuously for 16–20 weeks. The alkaloids were incorporated into the diets at the following levels: atabrine,[1] 0.02 per cent; quinine, 0.072 per cent; and auramine, 0.02 per cent. These levels represent a compromise between the toxicities of the compounds and their potencies as enzyme inhibitors (17); on a molar basis atabrine and auramine are approximately equal as enzyme inhibitors, while quinine is about ⅓ as effective. However, atabrine proved to be relatively toxic in the presence of the azo dyes with severe weight loss and death of some of the animals when 0.04 per cent, a level near the toxic level for young rats (16), was fed with the dye. Hence, 0.02 per cent was used in the actual tumor experiments. The level of auramine used was the molar equivalent of the higher concentration of atabrine.

After approximately 16 weeks on these diets, the extent of liver damage and neoplastic growth was determined by laparotomy. The animals were then either continued on the dye for several more weeks or they were fed the basal diet free from azo dye for a period of at least 10 weeks to permit any hepatomas induced by the carcinogen to grow to recognizable size. In most cases (all except groups 1–8, Table 1) the diet during this final period also contained the alkaloid fed previously.

Interrupted feeding of 3′-Me-DAB.—Male rats weighing approximately 200 gm. and female rats of approximately 175 gm. were fed diet I, containing choline chloride at a level of 1 gm/kg of ration and riboflavin at a level of 2 mg/kg. A diet containing 0.064 per cent of 3′-Me-DAB was fed for 4 weeks followed by 4 weeks of the dye-free basal ration with or without further alterations in diet (Table 2). The original diet containing dye was then fed for 4 more weeks, and, after the completion of this final dye-feeding period, the animals were continued on the basal ration free from dye for at least 12 more weeks. The compounds added to the basal ration during the 4-week middle period were atabrine, 0.04 per cent (in group 3 the level was lowered to 0.02 per cent after 2 weeks); urethan, 0.15 per cent; 2-acetylaminofluorene (AAF), 0.1 per cent; and DAB, 0.06 per cent. Methylcholanthrene was applied daily to certain animals as a 0.5 per cent solution in acetone.

EXPERIMENTAL

Tumors in rats fed atabrine, auramine, or quinine with DAB.—Atabrine fed continuously with DAB for 16–20 weeks failed to produce any consistent

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TABLE 1

THE EFFECT OF CERTAIN RIBOFLAVIN-ENZYME INHIBITORS ON THE PRODUCTION OF HEPATOMAS BY 0.06 PER CENT DAB

Group	Inhibitor	Av. initial wt. (gm.)	Av. wt. end dye feeding (gm.)	Weeks dye fed	Av. food consumption on dye (gm/rat/day)	Survival at end of dye feeding	No. tumors	Tumor-negative survivors	Per cent tumors
1	None	247	223	20	10.0	11/12	5	6	45
2	Atabrine	256	224		12.3	12/12	1	9	8
3	Quinine	240	230		11.4	11/12	5	4	45
4	Auramine	248	222		10.8	12/12	8	4	67
5	None	190	246	18	12.8	14/15	10	3	71
6	Atabrine	196	234		13.0	14/15	4	10	29
7	Quinine	193	250		13.9	15/15	3	12	20
8	Auramine	198	239		12.5	14/15	5	9	36
9	None	205	204	16	10.4	15/15	13	2	87
10	Atabrine	199	186		11.2	14/15	10	4	71
11	Quinine	201	192		10.4	15/15	15	0	100
12	Auramine	211	208		12.5	12/15	10	2	83
13	None	191	240	18	10.9	14/15	10	4	72
14	Atabrine	190	216		12.1	15/15	10	4	72
15	Quinine	191	240		13.5	15/15	8	4	67
16	Auramine	195	221		12.4	15/15	14	1	93

Groups 1–4 and 9–12 received a diet containing only 30 mg choline/kg; the diet fed the other groups contained 1 gm choline/kg. Groups 9–12 were female rats; the others were males.

TABLE 2

THE EFFECT OF ATABRINE, QUININE, AURAMINE, AND VARIOUS CARCINOGENIC COMPOUNDS ADMINISTERED BETWEEN TWO DOSES OF 3′-ME-DAB*

Group	Supplement during middle period	Sex	Av. initial wt. (gm.)	Av. wt. end 1st dye period (gm.)	Av. wt. start 2d dye feeding (gm.)	Av. wt. end of dye feeding (gm.)	Av. food consumption on dye (gm/rat/day)	Av. food consumption middle period (gm/rat/day)	Survival at end of dye feeding	No. tumors	Tumor-negative survivors	Per cent tumors
1	None	M	204	193	262	248	11.3	15.0	7/7	4	3	57
2	None	F	181	171	214	206	11.5	13.1	15/15	0	15	0
3	Atabrine 0.04 per cent	F	176	169	189	196	10.2	11.2	13/15	4	9	31
4	Quinine 0.096 per cent	F	182	182	221	204	10.8	13.6	15/15	0	15	0
5	Auramine 0.027 per cent	F	180	179	208	202	11.0	13.9	15/15	0	15	0
6	None	F	177	163	216	203	8.2	16.7	11/11	0	11	0
7	2-Acetylaminofluorene 0.1 per cent	F	178	165	169	184	9.0	10.5	9/11	3	6	33
8	Atabrine 0.04 per cent	F	200	179	191	180	7.7	12.7	7/9	2	5	27
9	None	M	225	204	287	283	14.3	13.8	11/12	3	8	27
10	Urethan 0.15 per cent	M	226	203	267	263	13.4	17.6	10/11	7	3	70
11	Methylcholanthrene† 0.5 per cent in soln.	M	222	201	284	281	14.0	17.6	9/11	1	8	11
12	2-Acetylaminofluorene 0.1 per cent	M	222	202	186	215	12.2	11.3	11/12	11	0	100
13	DAB 0.06 per cent	M	224	208	236	268	14.2	6.5	12/12	11	1	92
14	Atabrine 0.04 per cent	M	229	194	234	238	15.8	14.5	11/12	8	3	73
15	None	F	177	160	199	168	12.4	12.1	8/8	1	6	14
16	Urethan 0.15 per cent	F	169	155	187	177	12.9	11.9	7/8	2	4	33
17	Methylcholanthrene† 0.5 per cent in soln.	F	170	161	207	194	10.6	11.8	7/8	2	5	29
18	2-Acetylaminofluorene 0.1 per cent	F	170	159	153	154	15.5	11.6	10/10	5	5	50
19	DAB 0.06 per cent	F	174	157	170	161	9.9	8.8	8/8	4	4	50

* For the first 4 weeks of the experiment all groups were fed 0.064 per cent of 3′-Me-DAB; no dye was fed during the second 4 weeks, but the various groups received the supplements indicated; during the third 4-week period all groups received 3′-Me-DAB, and for 12 weeks thereafter basal ration was fed. The data in groups 15–19 were obtained by Ippolito Segre.

† Acetone solution painted on skin daily.

alterations in the number of tumors that developed. In two of the series the incidence of hepatic tumors was essentially the same in the groups fed atabrine as in the control groups (Table 1, groups 9 and 10, 13 and 14), whereas in two earlier series atabrine appeared to depress tumor development (Table 1, groups 1 and 2, 5 and 6). The latter result was not considered reliable, however, because of the high number of tumors in the control group 5 with respect to the other groups of the series (groups 6–8). In all other series quinine was without any effect on the development of hepatic tumors (Table 1, groups 1 and 3, 9 and 11, 13 and 15), while auramine appeared to increase tumor development somewhat in two of the four series (Table 1, groups 1 and 4, 13 and 16).

Tumors in rats fed atabrine or other substances alternately with 3′-Me-DAB.—Atabrine fed during a 4-week period of interruption between two periods of exposure to 3′-Me-DAB resulted in a consistent increase in the number of liver tumors that developed. In the first series (Table 2, groups 1–5), female rats fed the control diet failed to develop any tumors, although tumors appeared in four of fifteen receiving atabrine. No tumors developed when the supplements during the middle period were quinine or auramine. This series also illustrates the relatively great susceptibility of male rats to 3′-Me-DAB (29), since 57 per cent of the males on the control diet developed tumors, although none appeared in the corresponding females (Table 2, groups 1 and 2).

The effectiveness of atabrine in increasing the number of liver tumors was shown in a second series of female rats in which no tumors developed in the control group, although two developed in seven rats receiving 0.04 per cent of atabrine during the middle period (Table 2, groups 6 and 8). In fact, the incidence in the group fed atabrine was almost as high as that in a group receiving 0.1 per cent of the known carcinogen, 2-acetylaminofluorene, during the middle period (group 7).

The third series involved male rats, and here, too, the feeding of atabrine during the middle period resulted in a marked increase in tumor incidence—73 per cent of the rats developing tumors, as compared to only 27 per cent in the control group (Table 2, groups 9 and 14). In this series a number of carcinogens exerted an additive effect when fed alternately with 3′-Me-DAB. When DAB was fed between two doses of 3′-Me-DAB, the incidence of tumors was 92 per cent, as compared to 27 per cent in the control group (Table 2, groups 9 and 13). This agrees with the results of MacDonald *et al.* (21) that one azo dye can continue the carcinogenic process begun by another. When AAF was fed during the middle period (group 12), the incidence of tumors was 100 per cent; additive effects between azo dyes and AAF have also been observed previously (21). Urethan appeared to increase the incidence of tumors due to 3′-Me-DAB: from 27 per cent to 70 per cent in male rats (Table 2, groups 9 and 10) and from 14 to 33 per cent in females (groups 15 and 16). As far as we are aware, additive carcinogenic effects between azo dyes and urethan have not been observed previously (18, 26). Local application of methylcholanthrene to the skin during the middle period depressed hepatic carcinogenesis in males (groups 9 and 11) though not in females (groups 15 and 17). Others have observed a decreased development of liver tumors due to azo dyes when methylcholanthrene was fed (25) or applied intravaginally (28).

Storage and excretion of atabrine and quinine and effect on hepatic riboflavin.—Adult rats were fed various levels of DAB, atabrine, and quinine for periods of 5–7 weeks, and daily samples of urine and feces were collected at intervals during the final weeks of the experiment. The basal diet contained 2 μg of riboflavin/gm and the other ingredients of diet I. Atabrine, quinine, and riboflavin were then determined in urine and feces and in the livers at the end of the experiment. For these latter analyses livers from decapitated rats were homogenized with water in a Waring Blendor, one-half of the homogenates being used for the atabrine or quinine determination and the other half for the determination of riboflavin. The feces were homogenized in a similar manner. Extracts were prepared by established methods and the riboflavin content determined by titrating the acid produced by *Lactobacillus casei* on medium IV of Sauberlich and Baumann (6, 32) after 72 hours of incubation. The microbiological procedure was necessary, because preliminary experiments had indicated that atabrine, and, to some extent, quinine, interfered in the fluorometric determination of the vitamin (6). Recoveries of added riboflavin ranged from 95 to 110 per cent in the microbiological assay, and the levels of atabrine and quinine in the extracts were found to be much too low (6) to inhibit the assay organism (22).

For the determination of atabrine and quinine, 40 per cent of NaOH was added to the urine or to the liver or fecal homogenates, and the samples were heated on a water bath at approximately 90° C. for 30 minutes. The samples were then extracted with ether according to the method of Kelsey and Geiling (19). The ether layers were washed with 0.1 N KOH in 20 per cent ethanol and the compounds extracted from the ether layer with

0.1 N H_2SO_4. The concentrations of quinine and atabrine in the extracts were then measured fluorometrically (2, 19).

Very large amounts of atabrine, 11.7 mg., were found per liver, and the amounts of quinine were also appreciable—60–70 μg/liver (Table 3). When DAB was also fed, the amounts of atabrine and quinine in the liver were essentially the same as in the absence of carcinogen—10.5 mg. of atabrine and 103 μg. of quinine. More of the alkaloids were found in the urine than in the feces (Table 3), but the combined excreta and liver stores accounted for only a small fraction of the amounts ingested.

The feeding of 0.04 per cent of atabrine caused some decrease in the concentration of riboflavin in the liver, and a marked decrease in the amount per liver (Table 4). In fact, atabrine was almost as effective in decreasing hepatic riboflavin as the azo dye DAB (groups 2 and 6), and in rats fed DAB atabrine caused a further lowering in the riboflavin content of the liver (Table 4, groups 6 and 7, 16 and 17). Quinine and auramine, on the other hand, appeared to be without any consistent effect on hepatic riboflavin. All three alkaloids were without consistent effects on the amount of riboflavin excreted in urine (Table 4) or feces (6).

DISCUSSION

Although atabrine lowered the level of riboflavin in the liver, it does not appear that this was the mechanism by which the drug increased the

TABLE 3

HEPATIC STORAGE AND EXCRETION OF ATABRINE AND QUININE BY RATS INGESTING THESE SUBSTANCES

Group*	Diet	Amount of atabrine or quinine†		
		Total in liver (μg.)	Daily fecal excretion (μg.)	Daily urinary excretion (μg.)
1	Control	1–4 (AQ)	0–10 (AQ)	0–30 (AQ)
2	0.04 per cent atabrine	11,730 A	123 A	310 A
3	0.096 per cent quinine	60 Q	161 Q	444 Q
6	0.06 per cent DAB	0	0	0
7	0.06 per cent DAB+0.04 per cent atabrine	10,500 A	93 A	243 A
8	0.06 per cent DAB+0.096 per cent quinine	103 Q	202 Q	697 Q
15	0.096 per cent quinine	70 Q		
18	0.06 per cent DAB+0.096 per cent quinine	71 Q		

* The group numbers correspond to those in Table 4.

† A = atabrine; Q = quinine; (AQ) = apparent atabrine or quinine in control material; DAB = 4-dimethylaminoazobenzene.

TABLE 4

RIBOFLAVIN IN LIVERS AND URINE OF RATS FED ATABRINE, AURAMINE, QUININE, AND 4-DIMETHYLAMINOAZOBENZENE (DAB)

Group	Supplement Fed	Av. initial wt. (gm.)	Av. final wt. (gm.)	Av. food intake (gm/rat/day)	Liver riboflavin		Riboflavin in urine daily (μg)
					Total (μg)	Conc. (μg/gm)	
1	None	261	269	10.0	185	21.4	7.6
2	0.04 per cent atabrine	279	253	11.4	130	17.2	7.2
3	0.096 per cent quinine	296	309	11.0	199	21.1	4.8
4	0.027 per cent auramine	276	261	12.0	203	24.9	5.9
5	0.085 per cent auramine	293	212	9.9	228	30.6	3.4
6	0.06 per cent DAB	297	273	7.8	172	18.0	5.5
7	0.06 per cent DAB+0.04 per cent atabrine	295	204	11.8	107	13.1	7.9
8	0.06 per cent DAB+0.096 per cent quinine	269	229	7.8	139	19.8	4.8
9	0.06 per cent DAB+0.027 per cent auramine	287	238	9.0	125	16.3	4.6
10	None	201	247	14.6	193	24.0	9.6
11	0.02 per cent atabrine	198	230	14.8	159	22.0	
12	0.04 per cent atabrine	218	214	12.0	158	22.7	
13	0.04 per cent auramine	192	197	10.0	141	22.1	6.9
14	0.085 per cent auramine	219	182	9.9	161	24.8	9.0
15	0.096 per cent quinine	204	263	16.3	209	25.1	
16	0.06 per cent DAB	208	218	12.8	178	19.0	
17	0.06 per cent DAB+0.04 per cent atabrine	207	169	12.8	109	17.0	
18	0.06 per cent DAB+0.096 per cent quinine	206	205	12.3	132	16.5	
19	0.06 per cent DAB+0.027 per cent auramine	184	165	9.6	110	17.4	

Values are averages for three animals per group.

development of tumors due to the intermittent feeding of 3′-Me-DAB. The effectiveness of riboflavin in altering tumor development varies both with the azo dye and with the timing of the doses of dye and vitamin, respectively. Riboflavin is particularly effective in diminishing the carcinogenicity of DAB (20, 30), but atabrine did not enhance tumor development due to DAB in the present study (Table 1); and such variations in tumor incidence as occurred suggested an occasional delaying effect of atabrine. On the other hand, riboflavin is not particularly effective in preventing tumor development due to 3′-Me-DAB (11) and is even less effective when the vitamin is given between two periods of supplementation with 3′-Me-DAB (7). But these latter were the conditions under which atabrine was most effective in increasing tumor incidence (Table 2). The observed increases in tumor incidence would therefore appear to be associated with some liver-damaging action of atabrine other than that involving riboflavin directly.

Atabrine has been reported to produce gross liver necrosis and replacement fibrosis in rats fed 0.04 per cent of the drug for 180 days or 0.09 per cent for 35 days (23, 34, 35, 36, 38). Liver damage was relatively severe in older rats and was worse on a diet containing 6 per cent of casein than on one containing 30 per cent (35, 36), but the depression in growth, as compared to isocaloric controls, was essentially the same on the two levels of casein. Ershoff and others have reported that 0.03–0.05 per cent of atabrine is nontoxic on an adequate diet (8, 16) and that adverse effects of the drug may be minimized by whole liver, by a water-insoluble fraction thereof, by yeast, and, under certain circumstances, by vitamin B_{12} (8, 9).

Atabrine appeared to be noncarcinogenic in rats fed 0.01–0.08 per cent of the drug for 18 months (10) or in dogs fed 5–10 mg/kg of body weight for 1–6 months (15, 33). The increase in tumor development observed in the present study when atabrine was fed between two doses of 3′-Me-DAB (Table 2) would therefore seem to be more like the enhancing effect of croton oil on skin tumors due to hydrocarbons (3, 31) than the additive effects observed between related azo dyes or azo dyes and AAF (Table 2 and [21]). While liver-damaging regimens do not necessarily increase the carcinogenicity of azo dyes (7), an increased number of tumors has been observed when the diet fed between two doses of 3′-Me-DAB contained liver-damaging materials such as nicotinamide (7) or poor quality protein (yeast) (37).

The present results do not, of course, imply that any connection necessarily exists between atabrine and liver tumors in man. Many servicemen have taken about 0.06–0.1 gm. of atabrine daily for as long as 18 months without any indication of subclinical liver damage or even of impaired functional tests (1, 13, 24). These subjects, however, were presumably well nourished and without any particular susceptibility to primary cancer of the liver. On the other hand, adverse effects of atabrine might be possible in individuals such as the Bantu, many of whom are in a precancerous state (4, 12). Clark *et al.* (5) have already pointed out the inadvisability of atabrine therapy where liver or kidney damage exists.

SUMMARY

1. Atabrine, auramine, and quinine were without consistent effect on tumor incidence when the alkaloids were fed to rats with 4-dimethylaminoazobenzene (DAB).

2. Atabrine increased the incidence of liver tumors when fed between two doses of 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB). The increase was approximately equal to that observed when carcinogens such as DAB, 2-acetylaminofluorene, or urethan were fed between the two doses of 3′-Me-DAB. Quinine, auramine, or methylcholanthrene did not increase the incidence of liver tumors under these conditions.

3. The concentration of riboflavin was diminished in the livers of rats fed atabrine. The observed increase in tumors, however, appeared to be associated with some other (unknown) effect of atabrine upon the liver.

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Effects of A-Methopterin on Formate Incorporation into the Nucleic Acids of Susceptible and Resistant Leukemic Cells*

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The lack of cytotoxic specificity of the known anticancer agents and the development of drug resistance by originally inhibited neoplastic tissues have been causes of repeated disappointment in the field of cancer chemotherapy.

It seems likely that the usefulness of the presently known anticancer agents might be considerably increased if knowledge of the mechanisms of drug resistance in cancer could be obtained and successfully applied in its prevention. The development of sublines of mouse leukemia that are resistant to (2) or partially dependent on (4, 5, 7) antileukemic agents has provided a most useful tool for investigation of these mechanisms.

Drug resistance in micro-organisms has been known for many years and has been rather conclusively ascribed to chemical selection of existing drug-resistant mutants in a bacterial population. Evidence has been obtained which strongly suggests that the development of resistance in mouse leukemia is the result of a similar series of events (6).

It appears that the 4-aminopteroylglutamic acids prevent conversion of folic acid to citrovorum factor (8), a coenzyme essential to nucleic acid synthesis (12). The fact that desoxyribonucleic acid (DNA) will partially prevent the antileukemic action of A-methopterin (10) suggests that inhibition of nucleic acid synthesis is at least a part of the mechanism of action of citrovorum factor antagonists in neoplastic disease. It has been reported that formate incorporation into the nucleic acids of the livers and spleens of mice with a refractory strain of leukemia (Ak_4-R) was not inhibited to the same extent by A-methopterin as was observed in mice with a strain of leukemia which responds to treatment with this antagonist (11).

The present study was designed to obtain information on the effects of A-methopterin on nucleic acid metabolism in leukemic cells (L 1210) that are susceptible to inhibition by this compound and leukemic cells (L 1210-D) which on repeated transplantation in treated mice have become resistant and finally dependent on A-methopterin for optimal growth (5).

EXPERIMENTAL

The A-methopterin–dependent (L 1210-D) and susceptible (L 1210-S) strains of mouse leukemia used in these studies have already been described (5, 7).

The incorporation of formate-C^{14} into DNA guanine, adenine and thymine, and ribonucleic acid (RNA) guanine and adenine was measured at 6 hours. It is well known that formate is a precursor of the 2- and 8-carbon atoms of the nucleic acid purines and of the methyl carbon atom of thymine (14). Previous studies have shown that 4-aminopteroylglutamic acids profoundly inhibit incorporation of formate-C^{14} into nucleic acid purines of mouse viscera (12).

In order to compare the action of A-methopterin on nucleic acid synthesis in the susceptible leukemic strain and the dependent subline of L 1210 leukemia the following experiments were carried out: Groups of DBA mice received subcutaneous inoculations of susceptible or dependent leukemic cells and in certain instances were treated with A-methopterin and in others were left untreated. On the seventh day following leukemic inoculations, all mice were injected with 2.0 μc. each of formate-C^{14}. After 6 hours the animals were sacrificed, and leukemic tumors and viscera of the various groups were pooled and homogenized in a refrigerated Waring Blendor. Aliquots of the leukemic tumor homogenates and the viscera homogenates were then subjected to isolation procedures which provided small quantities of DNA and RNA (13). These nucleic acids were then hydrolyzed with perchloric acid, and the DNA guanine, adenine and thymine, and the RNA guanine and adenine were isolated with ion-exchange columns (Dowex-50). The actual amounts of the purines and thymine obtained were determined spectrophotometrically, and carrier was added in known amounts to facilitate isolation from ion-exchange column eluates. Corrections were then applied in calculations of specific activities based on the known dilution factors.

The details of the treatment of animals and the results ob-

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tained are presented in Table 1. The column "initial homogenate" refers to activity data on the whole tissue indicated.

DISCUSSION

Results presented in Table 1 show that formate incorporation into DNA guanine, adenine and thymine, and into RNA guanine and adenine of the sensitive L 1210 leukemia and viscera is profoundly inhibited by A-methopterin. This was not unexpected in view of previous observations (12). The most interesting result was that A-methopterin treatment caused an increase in formate incorporation by the A-methopterin–dependent leukemia and at the same time inhibited formate incorporation into the visceral nucleic acids of animals bearing these leukemic tumor masses. These data clearly indicate a different metabolic response of the dependent leukemia and the viscera to A-methopterin.

TABLE 1

THE EFFECT OF A-METHOPTERIN ON FORMATE INCORPORATION INTO NUCLEIC ACID MOIETIES OF SUSCEPTIBLE AND DEPENDENT LEUKEMIC CELLS

Group	No. of mice	Tissue	Treatment	Specific Activities (μc/mole C) Initial homogenate	DNA Guanine	DNA Adenine	DNA Thymine	RNA Guanine	RNA Adenine
1	15	Leuk. L 1210-S	None	4.2	206	227	100	309	308
		Viscera	None	6.9	171	280		199	248
2	15	Leuk. L 1210-S	A-meth.*	1.8	34	39	10	54	112
		Viscera	A-meth.*	5.6	26	16	4	70	90
3	16	Leuk. L 1210-D	None	1.8	55	51	24	85	86
		Viscera	None	7.3	113	99		156	140
4	15	Leuk. L 1210-D	A-meth.*	4.8	127	115	51	162	156
		Viscera	A-meth.*	4.9	15	10	3	37	35

* A-methopterin (3 mg/kg) was administered intraperitoneally immediately before injection of formate-C^{14} on the 7th post-inoculation day.

† A-methopterin (3 mg/kg) was administered intraperitoneally on the 1st, 3d, 5th, and 7th post-inoculation days. Formate-C^{14} was injected immediately after the last A-methopterin injection on the 7th day.

Note: 2 μc. of formate-C^{14} was injected/20-gm mouse. All experiments were terminated at 6 hours after formate injection. Viscera include livers, spleens, small intestines, kidneys, and testes. Average leukemic tumor weights for groups 1, 2, 3, and 4 were: 670, 670, 700, and 930 mg., respectively.

The questions whether nucleic acid metabolism is the primary site of the anti-leukemic action of A-methopterin and whether mutations which result in a failure of A-methopterin to inhibit formate utilization in resistant cells are largely responsible for A-methopterin resistance are still lacking direct evidence. In support of these interpretations are observations that (*a*) thymidine (a moiety of DNA) will prevent the toxicity of 4-aminopteroylglutamic acids in bacteria (1); (*b*) DNA will partially prevent the antileukemic action of A-methopterin (10), while serine, glycine, methionine, and formate are inactive in this respect;[1] (*c*) A-methopterin causes a build-up of 4-amino-5-carboxamidoimidazole in *E. coli* (15). However, it should be pointed out that the inhibition of formate incorporation into the whole tissue homogenates caused by A-methopterin in these experiments cannot be accounted for on the basis of inhibition of nucleic acid synthesis, unless one assumes that inhibition of nucleic acid synthesis is followed by failure of the cells to incorporate formate into protein. Only about 10 per cent of the formate carbon in the initial homogenates can be accounted for in the nucleic acid bases.

One of the hypotheses which is sometimes offered to explain drug resistance is the existence of alternative pathways of making inhibited products. The present results show that formate is utilized by the A-methopterin–dependent strain of leukemia in *de novo* synthesis of nucleic acid purines and thymine. Also, it is clear that A-methopterin in some way stimulates this anabolic process as well as formate incorporation into cell fractions other than nucleic acids in the dependent leukemia (see initial homogenates in leukemia L 1210-D, groups 3 and 4).

The fact that citrovorum factor will partially prevent the growth-promoting action of A-methopterin for the L 1210-dependent leukemia (4) makes untenable a number of possible explanations of the mechanism of resistance to or dependence on this compound. However, these phenomena might be explained on the basis of differences in the geometry of the apoenzyme for which A-methopterin and citrovorum factor (CF) compete, resulting in a stronger binding of CF in the dependent strain (3). This explanation requires the assumption that dependence is the result of too tight binding of citrovorum factor to an apoenzyme and that A-methopterin stimulates growth in this case by correction of the dissociation constant to optimal for leukemic cell growth. This is an attractive postulate which fits in with certain observations on bacteria, i.e., "more and more it is being realized that the genetic blocks of enzymatic

[1] H. E. Skipper, M. Bell, and J. Chapman, unpublished data.

reactions involve inhibitions by normal metabolic products of the organism. These inhibitions are not unrelated in character to those obtained with synthetic analogs of metabolites" (9).

A second possible explanation of dependence is that A-methopterin inhibits the excessive production of a normal metabolite. If one assumed that CF was not the final formylating coenzyme but was intermediate en route to this coenzyme (CF^1), then the following might be suggested as a means of fitting the present facts together: The dependent strain might be deficient in the enzyme necessary for conversion of $CF \rightarrow CF^1$. Thus, there would be a build-up of CF in the untreated dependent cell which might compete with CF^1, thus inhibiting growth. If A-methopterin inhibited the conversion of folic acid to CF in the dependent strain, this would depress the competing CF pool and thus promote growth of the dependent leukemic cells. Likewise, administration of CF would be expected to prevent the growth promotion of A-methopterin in the dependent leukemia.

The present data are compatible with the thesis that A-methopterin promotes activation of the enzyme systems involved in formate transfer or prevents some natural inhibition of these enzymes. Further work on these questions is in progress.

SUMMARY

It has been demonstrated that A-methopterin inhibits formate incorporation into nucleic acid purines and thymine in leukemic cells which are sensitive to this compound and also in viscera of mice bearing these susceptible leukemic cells. A-methopterin, however, causes a significant increase in formate incorporation into the nucleic acids of leukemic cells which have become dependent on A-methopterin for optimal growth while inhibiting formate incorporation in the visceral nucleic acids of mice bearing the dependent strain of leukemia.

Possible mechanisms involved are discussed.

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Effect of Irradiation on Lymphoid Tissue Nucleic Acids in C57BL Mice*

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Lymphoid tumors of thymic origin are induced in a high percentage of C57 black mice (C57BL) exposed to periodic whole-body x-radiation (10). To explore the biochemical basis of this phenomenon, study of nucleic acids in normal and irradiated thymus and lymph nodes was undertaken.

Nucleic acids were chosen for study because of evidence suggesting that they may be important in protein synthesis (5, 7, 8). Moreover, there have been reports of differences between nucleic acid concentration in tumor tissues and the homologous normal tissues (13, 14, 16) and of disturbances in nucleic acid concentration following x-radiation (4, 9, 12, 15). It seemed likely, therefore, that changes in concentration of these compounds in irradiated lymphoid tissues, possibly related to the process of tumorigenesis, might be demonstrated.

MATERIALS AND METHODS

C57BL mice of both sexes were assigned to control or experimental groups of 6–15 animals each, designated for autopsy at serial time intervals. In the experimental groups irradiation was begun when the mice were 33 ± 3 days old and was administered in four whole-body exposures of 168 r each at 8-day intervals.[1] Animals were maintained under identical laboratory conditions with free access to Purina Laboratory Chow and water. After the appropriate intervals, control and irradiated litter-mate groups were killed with ether. The thymus and superficial lymph nodes (two axillary, two inguinal) were rapidly dissected free of fat and connective tissue, weighed on a torsion balance, and immediately frozen in dry ice. These tissues, weighing 3–50 mg., were then ground with iced, distilled water in chilled, glass micro-homogenizers and transferred to 5-ml. centrifuge tubes with a total volume of about 1 ml. of cold, distilled water. Nucleic acids were separated by a modification of the method of Schmidt and Thannhauser (6). This procedure was further modified in that the initial acid separation and washing were carried out in the cold; and lipids were extracted by treatment with 95 per cent alcohol, followed by ether-alcohol (1:3) at about 60° C. After digestion with perchloric and nitric acids, aliquots of each sample were analyzed for phosphorus by the micro-method of Berenblum and Chain (3), modified by the use of digestion tubes for partition, mixing with filtered air, and removing the aqueous layer by suction. The amount of phosphorus per sample was calculated from the spectrophotometric density at 630 mμ. From this value, the phosphorus content of the precipitated desoxyribonucleic acid fraction (DNAP) and that of the supernatant fraction (RP) containing ribonucleic acid, phosphoprotein, and perhaps other unidentified phosphorus-containing compounds were determined and expressed as μg P/mg wet weight of tissue.[2]

TABLE 1

NUCLEIC ACID CONCENTRATIONS IN LYMPHOID TISSUES OF C57BL MICE

Exp. day	Age in days	Group	No. mice	Body wt. (gm.)	Thymus Wet wt. (mg.)	Thymus μg P/mg wet wt DNAP*	Thymus μg P/mg wet wt RP*	Lymph nodes Wet wt. (mg.)†	Lymph nodes μg P/mg wet wt DNAP*	Lymph nodes μg P/mg wet wt RP*
	35	Control	5	17.9	57.6	2.39 ±0.063		3.12	1.11	
29	62	Control	9	20.1	50.2	3.13 ±0.082	0.71 ±0.019	3.58	2.07‡±0.097	0.71‡±0.052
		X-ray	8	17.8	14.5	1.65 ±0.046	0.79 ±0.033	3.90	1.26 ±0.056	0.65 ±0.021
57	90	Control	6	21.5	45.0	3.76‡±0.23	0.76 ±0.032	2.84	2.25‡±0.12	0.68 ±0.022
		X-ray	6	21.1	17.7	2.30 ±0.10	0.82 ±0.020	1.82	1.43 ±0.14	0.71 ±0.031
86	120	Control	5	24.2	35.5	3.22‡	0.76 ±0.021	4.32	1.95 ±0.0076	0.67 ±0.022
		X-ray	6	23.5	25.5	2.11 ±0.098	0.92 ±0.042	4.23	1.60‡	0.76‡
115	149	Control	15	25.3	32.8	2.95 ±0.084	0.68 ±0.032	3.67	1.91 ±0.026	0.59 ±0.015
		X-ray	14	23.4	34.7§	1.91 ±0.012	0.94 ±0.036	7.00§	1.61 ±0.059	0.66 ±0.028
142	174	Control	17	25.3	32.9	2.62 ±0.077	0.62‡±0.017	3.96	1.80 ±0.048	0.52‡±0.019
		X-ray	15	22.5	98.7§	1.89 ±0.092	0.76 ±0.033	7.45§	1.61 ±0.095	0.61 ±0.052

* Values are average ± standard error.
† Average weight of one node.
‡ One or two samples lost before analysis.
§ Some of these tissues were involved by lymphoma.

[1] Physical factors were: 120 kvP; 9 ma.; target-mouse distance, 30 cm.; 0.25 mm. Cu+1.0 mm. Al added filter, output 31 r/min.

[2] Recoveries of 1 and 3 μg. DNA added to tissue homogenate were 99 and 101 per cent, respectively. Recovery of added RNA was 80 per cent.

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RESULTS

The data are summarized in Table 1 and Charts 1 and 2.

Normal lymphoid tissues.—DNAP concentration in normal animals increased with age to a maximum at 90 days in both thymus and lymph nodes, followed by a gradual and progressive decrease through 174 days. There was no significant change in RP concentration during the period of observation. Andreasen and Ottesen (2) had previously noted a similar change in nucleic acid concentration with age in rat thymus.

Normal thymus DNAP concentration was consistently higher than that of lymph nodes at all

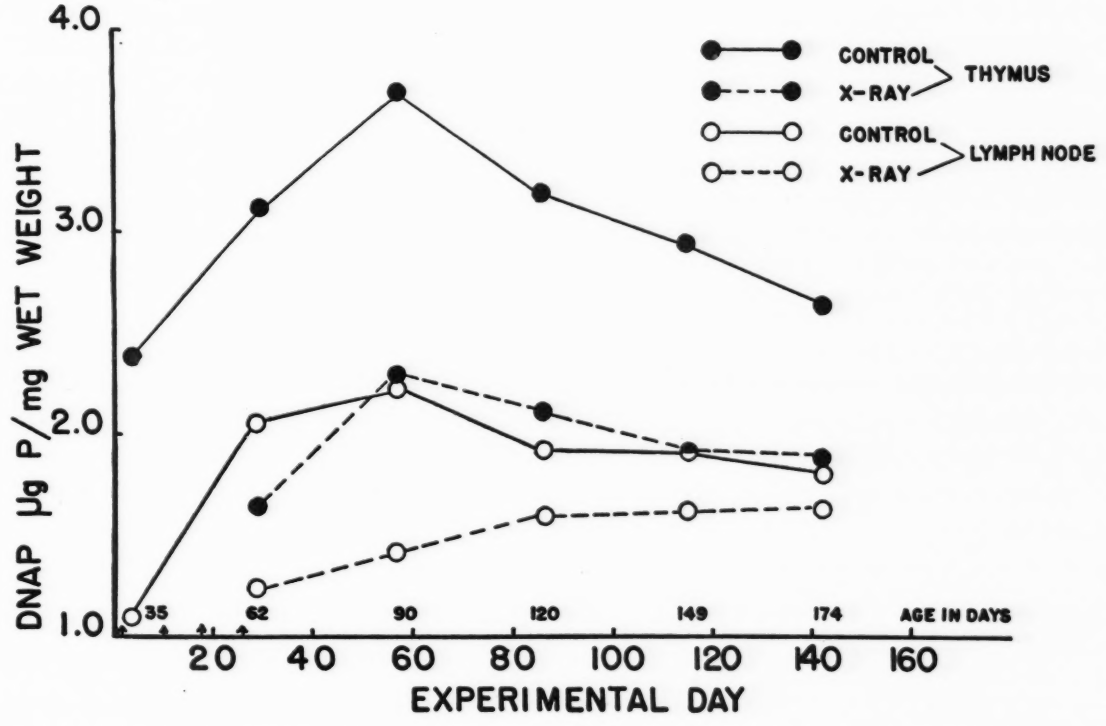

CHART 1.—DNAP concentration in lymphoid tissues of normal and irradiated C57BL mice

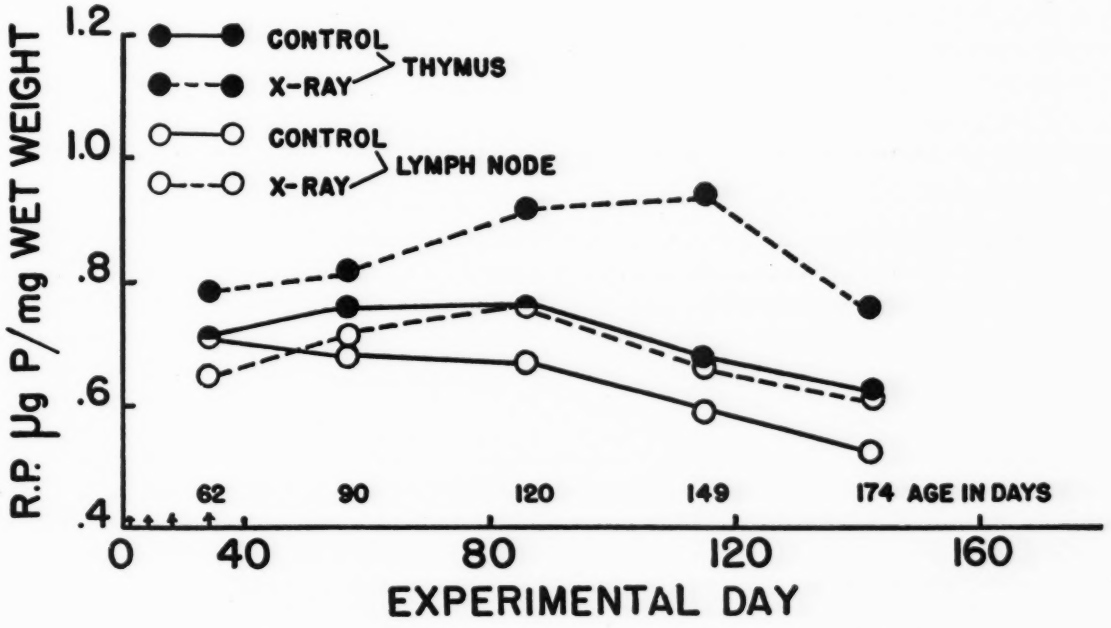

CHART 2.—RP concentration in lymphoid tissues of normal and irradiated C57BL mice

time intervals studied, in ratios ranging from 1.45 to 2.16, with a mean of 1.67. In contrast, average RP concentrations in lymph nodes and thymus were not significantly different in any group, nor in the pooled data.

Irradiated lymphoid tissues.—Within 4 days after the last treatment (day 29), mean DNAP concentration had dropped to 53 per cent of normal in thymus and 61 per cent in lymph nodes (Table 2). Although the concentration increased somewhat thereafter in both irradiated tissues, it remained considerably below control levels in the thymus, whereas in the lymph nodes it was no longer significantly reduced by experimental day 142. At days 115 and 142, there were no apparent differences in DNAP concentration among thymuses of the test groups, despite morphological differences ranging from involutional changes to well established lymphomas (Table 3).

RP concentrations were slightly elevated after irradiation (averaging about 20 per cent above pooled normal levels in thymus, 12 per cent in lymph nodes), but the group differences were not statistically significant except for thymus at days 86 and 115.

TABLE 2

DECREASE IN DNAP CONCENTRATION IN LYMPHOID TISSUES OF C57BL MICE AFTER X-RAY

Experimental day	29	57	86	115	142
			μg P/mg wet wt: Thymus		
Control	3.12	3.76	3.22	2.95	2.62
X-ray	1.65	2.30	2.11	1.91	1.89
Per cent difference	47.1	38.8	34.5	35.4	27.8
			μg P/mg wet wt: Lymph nodes		
Control	2.07	2.25	1.95	1.91	1.80
X-ray	1.26	1.43	1.60	1.61	1.61
Per cent difference	39.2	36.4	17.9	15.7	not sig.

DISCUSSION

Since lymphoid tumors which develop in irradiated C57BL mice show a striking tendency to arise in the thymus, it might be expected that chemical changes of particular significance in the induction process would either occur in the thymus alone or would differ in this organ from responses in other lymphoid tissues. It is provocative to note, therefore, that thymic DNA in this strain differs from that of the superficial lymph nodes in at least two aspects: normal concentration and response to irradiation. Thymic DNAP concentration is consistently higher than that of lymph nodes, while RP concentration is the same in both tissues. Andreasen (1) has previously reported a higher concentration of nucleic acid phosphorus in thymus than in lymph nodes of rats.

Following irradiation, DNAP concentration decreased markedly within 4 days in both tissues but gradually returned to normal only in lymph nodes during the period covered by this investigation. In general agreement with the present data, other investigators have noted that DNAP concentration (*a*) decreased in rabbit lymphoid tissues within 24 hours after 500–1,000 r whole-body irradiation (4); (*b*) diminished 57 per cent in rat thymus 3 days after a single LD_{50} dose of P^{32} and remained so through 8 days (11); and (*c*) was reduced in the spleens of mice with spontaneous leukemia (13), though to a lesser extent than noted in thymic lymphomas induced by irradiation in the present study.

However, the relative lack of change of RP values is in conflict with Mitchell's observation (12) of a large increase in cytoplasmic pentose nucleotides of various tissues after x-radiation. Moreover, Stowell (16), using a semi-quantitative microspectrophotometric method, found either normal or slightly increased amounts of DNA per nucleus in 31 tumors, as compared to their normal homologous tissues. These and other considerations indicate that determinations of nucleic acid per cell and of DNA per nucleus are essential for an interpretation of the chemical data.

In the light of Swift's demonstration (17) that normal mouse lymph nodes and thymus each contain two cell populations, one with twice the amount of DNA per nucleus than the other, it might be postulated that irradiation effects a decrease in DNA concentration by selective destruction of the DNA-rich cells. Histologic examination of these tissues at intervals after irradia-

TABLE 3

HISTOLOGY OF THYMUS OF C57BL MICE 3–4 MONTHS AFTER X-RAY COMPARED TO DNAP CONCENTRATIONS

Experimental day	Animal no.	DNAP concentration*	Histology
	26	1.63	Lymphoma†
	42	2.11	Very early lymphoma, one lobe
115‡	48	2.17	Very early lymphoma, both lobes
	70	1.57	Localized lymphoma
	72	2.30	Hyperplasia
	76	2.21	Marked involution
	78	2.24	Marked involution
142§	90	1.66	Marked involution
	2	1.25	Early lymphoma, one lobe
	8	1.69	Early lymphoma, one lobe
	34	2.06	Questionable lymphoma

* μg P/mg wet wt.

† This animal also showed involvement of lung, kidney, liver, and spleen.

‡ Other DNAP values, day 115: range, 1.29–2.88; average, 1.91 ± 0.12.

§ Remaining animals with highly invasive lymphoma gave DNAP values of 2.27, 2.32, 2.06, 1.37, 1.45, 1.83, 2.38, 2.03, and 1.78 μg P/mg wet wt.

tion reveals a striking succession of changes. Within a few days, the thymus reveals a profound depletion of lymphocytes, particularly in the cortex, and similar changes are found in lymph nodes. Repopulation of these tissues and reconstitution of their architecture occur within about 30 days, and lymphoid tumors appear in some instances by experimental day 115. Since thymic DNAP concentration remains significantly reduced throughout this cycle of injury, repair, and neoplasia, it is difficult to perceive any simple correlation with tissue cellularity or other morphological changes.

SUMMARY

In agreement with earlier observations, it was found that desoxyribonucleic acid phosphorus (DNAP) concentration increased with age in thymus and lymph nodes of normal C57BL mice, reaching a maximum at 90 days and decreasing thereafter. Concentration was higher in thymus than in lymph nodes at all ages studied.

The concentration of phosphorus in the fraction (RP) containing ribonucleic acid did not change appreciably with age and was the same in thymus and lymph nodes.

In C57BL mice treated systemically with four doses of 168 r at 8-day intervals, thymic concentration of DNAP decreased 40 per cent 4 days after treatment and remained significantly reduced through 142 days, at which time most of the thymuses were involved by lymphomas. In contrast, lymph node DNAP concentration, although depressed immediately after irradiation, returned to normal by 117 days after treatment. Irradiation resulted in a slight increase in RP concentration in both tissues when pooled data were examined.

Interpretation of these findings will require correlation with microphotometric determinations of DNA per nucleus in normal and irradiated lymphoid tissues.

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Announcements

The International Union against Cancer

(A Report of the U.S.A. National Committee on the International Union against Cancer of the National Research Council)

The first attempt to create a world-wide Cancer Organization was made before World War I under the auspices of "The International Association for Cancer Research." This organization was officially cancelled when Professor J. Fibiger of Copenhagen, Denmark, resigned as president of the 4th International Congress which was to be held in Copenhagen at the beginning of the first world war. This association had been very active. Three international Congresses were organized (1907, 1910, and 1913), and an active international campaign was carried out to educate the public in matters dealing with cancer.

Following World War I, the first sign of the revival of international activity in the field of cancer was the organization of a conference at Strasbourg, in 1923, on the occasion of the centennial anniversary of the birth of Pasteur.

Three years later, in 1926, Doctor F. L. Soper, then Secretary, and Doctor Howard C. Taylor, President of the American Society for the Control of Cancer, organized an international symposium at Lake Mohonk, New York, U.S.A., which was attended by representatives of eight nations. This symposium, a model of its kind, was a great success.

In 1928 the British Empire Cancer Campaign organized another international conference in London, attended by representatives of sixteen nations. Discussions were held regarding dissemination of cancer information to the public, and for the first time the problem of the geographic and racial distribution of cancer was discussed.

A successful attempt to establish a permanent international group after World War I (which had been suggested at the meeting in America in 1926) was made in Madrid, Spain, in 1933 by the French Minister of Health, Justin Godart, and Doctor Jacques Benadaline. This first meeting of the newly formed International Congress against Cancer was concerned with the scientific aspects of cancer as well as the control aspects. As a result, the International Union against Cancer (Union Internationale Contre le Cancer, UICC) was created in 1933 with Godart as president, Paris as the headquarters and the by-laws registered under French law. The second Congress organized by the Union was held in Brussels, Belgium, in 1936 and the third in Atlantic City, New Jersey, U.S.A., in 1939.

The accomplishments of these international groups up to this time have been the organization of Cancer Congresses; the establishment of an international journal, Acta Unio Internationalis Contra Cancrum, edited by Professor J. Maisin, Voer des Capucins, 61, Louvain, Belgium, to publish the proceedings of the Congresses; the encouragement of studies in Geographic Pathology; the international dissemination among scientists and the public of information dealing with cancer; and some recommendations for a central abstracting center for cancer literature, for the preparation of standard cancer nomenclature, and for atlases of tumor pathology. Recently, in the United States, the American Cancer Society has published its *Manual of Tumor Nomenclature and Coding*, which is now in the Statistical Committee of WHO for international adoption. The *Atlases of Tumor Pathology* are currently in preparation under the auspices of the Subcommittee on Oncology of the National Research Council.

During World War II, all activities of the International Union against Cancer ceased. Following the war, the Union accepted the invitation of the American Association for Cancer Research to hold the 4th International Cancer Research Congress in St. Louis, U.S.A., in 1947, and Doctor E. V. Cowdry was selected as president. The 5th Congress was held in Paris, France, in 1950, and the 6th is scheduled to be held in São Paulo, Brazil, in January, 1954.

The International Union against Cancer has been represented in the World Health Organization (WHO) and in the United Nations Educational, Scientific and Cultural Organization (UNESCO) through the Council for International Organization of Medical Sciences (CIOMS).

The International Cancer Research Commission.—At the St. Louis meeting of the Union in 1947, the International Cancer Research Commission (ICRC) was created as an outgrowth of the Scientific Committee of the Union. This move reflects the expanding activity in the field of cancer research throughout the world. The Commission has greater autonomy within the Union than had the Scientific Committee. The principles of the Commission as originally stated embraced all efforts to advance our knowledge of cancer by clinical and experimental means. The Commission as an advisory body will help to plan and promote cancer research, and it is largely responsible for developing the field of geographic pathology, for standardization of the nomenclature for cancer, and for indexing of cancer literature, all at the international level. The first president of the Commission was Doctor I. Milan of Mexico, and the current president is Professor V. R. Khanolkar of Bombay, India. The last meeting of the Commission was held in Lisbon, Portugal, on December 15–20, 1951. The Executive Committee of the International Union against Cancer met at the same time also in Lisbon.

Summary of the Lisbon meetings, December, 1951.—Because some important decisions were made and some clearly defined resolutions were offered at the meeting of the Executive Committee of the Union and the meeting of the Scientific Commission (International Cancer Research Commission) at Lisbon, Portugal, in December, 1951, a summary of this meeting is presented.

In the Executive Committee of the International Union against Cancer it was proposed to create within the structure of the Union an international committee to concern itself with problems relating to the control of cancer. This was voted on and passed unanimously. The Executive Committee appointed the following members to draft the aims, objectives and duties of such a committee.

Professor Xenophon Chahovitch, Belgrade, Yugoslavia.

Professor J. H. Maisin, Institut du Cancer, Louvain, Belgium.

Professor J. L. Nicod, Université de Lausanne, Lausanne, Switzerland.

Professor Nicholas Puente-Duany, de L'Institut du Radium, Cuba.

Dr. Charles S. Cameron, American Cancer Society, New York, New York, U.S.A.

The Executive Committee realized that to insure a more effective functioning of the UICC, greater financial stability was desirable. It was voted that participating countries and organizations should be approached and a plea made for an increase in the contributions to the Union.

At the Lisbon meeting, the ICRC elected a committee on Geographic Pathology and one on Nomenclature, approved a bibliographic center for indexing and documentation of cancer literature, and adopted the following resolutions primarily concerned with the membership of the Commission and future meeting dates:

1. That National Representatives to the Commission should be nominated by their governments with the advice and support of either cancer research organizations or medical faculties.
2. That the program of the meetings will be drawn up by the Executive Committee of the International Cancer Research Commission.
3. That one member of the Executive Committee shall be appointed as Secretary of the ICRC for the period of his tenure on the Executive Committee.
4. That there shall be created a new category of membership, associate member, to be nominated upon the recommendation of the Chairman of the Committees; these members are invited to take part in the work of the committees and in the meetings of the Commission, but shall not be entitled to vote.
5. That the International Congress on Cancer at São Paulo, Brazil, shall commence on January 3, 1954.
6. That the date of meetings of the Executive Committee of the Union and of the ICRC shall be fixed as December 28, 1953.
7. That the next meeting of the ICRC shall be in Bombay, India, in December, 1952, and that in the future only one meeting will be held during the intervening period between two International Congresses.
8. That the annual subsidy granted by the Union to the Commission shall be raised from its present level of $1,200 to $2,400.

The headquarters of the Sub-Committee on Geographic Pathology was established at the National Cancer Institute, Bethesda, Maryland, U.S.A., and the following members were elected to the Committee:

Professor Johannes Clemmesen, Associate Secretary, The Danish Cancer Registry, Copenhagen, Denmark.

Doctor Harold L. Stewart, Executive Secretary, Chief, Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland, U.S.A.

Professor V. R. Khanolkar, Ex officio, Executive Officer, Tata Memorial Hospital, Bombay, India.

Doctor Pierre Denoix, National Institute of Hygiene, Paris, France.

Professor Joseph Gillman, Medical School Hospital, Johannesburg, South Africa.

Professor J. H. Maisin, Institut du Cancer, Louvain, Belgium.

The following members were elected to the Sub-Committee on Nomenclature:

Doctor Isabella H. Perry, Chairman, University of California Medical School, San Francisco, California, U.S.A.

Professor E. Berven, Radiumhemmet, Stockholm, Sweden.

Doctor Pierre Denoix, National Institute of Hygiene, Paris, France.

Professor Herwig Hamperl, l'Institut Pathologique, Marburg Lahn, Germany.

Doctor Cecil Jackson, Medical School Hospital, Johannesburg, South Africa.

Professor Mathias Roxo-Nobre, Clinico da Clinica da Associacao Paulista de comb. ao Cancer, São Paulo, Brazil.

Doctor C. Chester Stock, Sloan-Kettering Institute for Cancer Research, 410 East 68th Street, New York, New York, U.S.A.

It was proposed: that a World Cancer Day be observed and that all necessary measures be taken to insure its success; that the issuance of an international stamp for the campaign against cancer be approved; and that the issuance of a special stamp for future International Congresses in the countries in which they will be held be approved.

The two sub-committees of the ICRC mentioned above reported to the Commission at the Lisbon meetings, and some of the points discussed and agreed upon are of sufficiently broad interest to warrant presentation here:

Sub-Committee on geographic pathology.—The Sub-Committee on Geographic Pathology has defined its field of interest as comprising knowledge concerning variations in the distribution and behavior of cancer among various ethnologic groups in different localities in relation to local factors.

This Sub-Committee agreed that its primary functions would be:

1. To serve as a point of contact and communication between investigators in its field of interest throughout the world.
2. To invite the co-operation of cancer investigators to serve as correspondents in order that centers of information in the various countries of the world might be established.
3. To encourage and facilitate co-operation on specific scientific problems by assisting in the procurement of grants-in-aid for research.
4. To call small groups of experts together for symposia concerned with particular problems in the geographic pathology of cancer.
5. To promote the publication of information on the endemiology of cancer in journals devoted to cancer research.

Sub-Committee on tumor nomenclature.—Dr. Isabella Perry, Chairman of the Sub-Committee on Tumor Nomenclature, had forwarded to members of the Committee a provisional draft of the *Manual of Tumor Nomenclature and Coding* published by the American Cancer Society. The members had voted to approve the manual and the Committee recommended that, for the present, work on a code for the clinical stages of carcinoma be confined to accessible sites. It was agreed that the criteria for determining the clinical stages of carcinoma for accessible sites would be the physical examination supplemented by such instrumentation as is in general use by the practitioner and not such as to require consultation with a specialist. The following general code for the clinical stages of accessible carcinoma was proposed:

Primary site:

O No tumor.
I Tumor (relatively larger size), infiltrating the primary organ site.
II Tumor of relatively larger size, infiltrating the primary organ site and not extending beyond the adjacent soft tissues.
III Tumor widely infiltrating primary organ site and extending to neighboring organs.
IV Tumor extensively involving adjacent soft tissues or organs.

Metastases:

O None.
I Regional lymph nodes slightly enlarged, discrete and movable.
II Regional lymph nodes considerably enlarged, discrete and movable.
III Regional lymph nodes enlarged, and fixed either to one another or to adjacent structures.
A Clinical stages confirmed microscopically.

This Committee felt that there is considerable evidence of a need for a clearer and more uniform nomenclature and recording for tumors. It was pointed out that WHO has recommended the establishment of a "clearing center for problems arising in the application of the *International Statistical Classification*" and a "focal unit" for maintaining relationships with national committees or other organizations of national scope. The Committee recommended approval of the proposed "clearing center" and "focal unit" and entered a plea to be sent to the Chairman of the WHO sub-committee on the *Registration of Cases of Cancer as Well as Their Statistical Presentation* for the prompt establishment of at least the cancer sections of these offices.

United States National Committee on International Union against Cancer.—As a result of the increased activity of the International Union against Cancer since World War II, there has been a growing feeling on the part of individuals and organizations in the United States with an interest in cancer that some group should be formed to organize, co-ordinate, and strengthen the participation of the United States in the International Union. In April, 1951, Dr. William U. Gardner was selected for the second time to serve as American Representative to the International Research Commission of the Union by the American Association for Cancer Research. Dr. Gardner requested and received authorization of this group to discuss with other organizations interested in cancer the suggestion that representation of the United States in the Union be formalized through the establishment of a perpetuating organization affiliated with the National Research Council of the United States.

This suggestion was agreeable to individuals and agencies interested in cancer and in the Union. As a result, a meeting of representatives of the American Association for Cancer Research, the American Cancer Society, the International Union against Cancer, the National Cancer Institute, and the National Research Council was called on October 21, 1951, to consider ways and means of establishing a group to facilitate representation and participation of the United States in the Union. At this meeting the National Research Council was requested to establish the National Committee on the International Union against Cancer consisting of at least five members selected from nine nominations, three each of which would be submitted by the American Association for Cancer Research, the American Cancer Society, and the National Cancer Institute. Each member would serve for a period of 5 years, except that the original members would be appointed for staggered terms so that thereafter one new member would be appointed each year from nominations submitted by the three agencies mentioned above.

It was agreed that the general functions of this Committee would be:

1. To select and instruct representatives to meetings of the Union and Commission and to receive reports from these representatives.
2. To devise means for the support of the functions of the Committee, its delegates, and representatives in relation to the general and specific functions.
3. To establish an office or facilities for the transmission of information relating to cancer to the Union or Commission or its members in the several countries and to receive similar material for appropriate distribution in the United States.
4. To approve all groups from the United States that may apply for membership in the Union and other-

wise to encourage membership in the Union so as to foster its activities.

Subsequently the National Research Council agreed to establish the National Committee on the International Union against Cancer in the Division of International Relations and in close liaison with the Division of Medical Sciences. The following members have been appointed.

Dr. William U. Gardner, *Chairman*, Yale University.
Dr. Charles S. Cameron, American Cancer Society.
Dr. E. V. Cowdry, Washington University.
Dr. John R. Heller, National Cancer Institute.
Dr. Paul E. Steiner, University of Chicago.

At its first meeting on March 3, 1952, the Committee accepted and approved the report by Dr. Harold L. Stewart, National Cancer Institute, who was the official representative of the United States at meetings of the Union and the Commission in Lisbon, Portugal, on December 15–20, 1951. The Committee also drafted a Constitution and discussed the selection and instruction of delegates to meetings of the Union and ways of obtaining financial support for the Union. The Committee has had two subsequent meetings, and at its latest on June 16, 1952, plans were made for the participation of six delegates from the United States in a conference on the "Endemiology of Lung Cancer" to be held in Louvain, Belgium, on July 21–23, 1952. The Committee also appointed Dr. Harold L. Stewart of the National Cancer Institute to represent the United States at the next meeting of the International Cancer Research Commission to be held in Bombay, India, in December, 1952, or in January, 1953.

There are three categories of membership within the Union—namely, countries, private organizations, and individuals—requiring membership fees of $250.00, $100.00, and $10.00, respectively. It has been agreed with the Union that membership in the Union from the United States shall be approved by the National Committee. The members of the Committee are anxious to do everything possible to increase interest in and support of the International Union against Cancer. Questions, suggestions, or requests for further information should be directed to the National Committee on the International Union against Cancer, National Research Council, 2101 Constitution Avenue, Washington 25, D.C.

AEC Cancer Program for Fiscal Year 1953

The U.S. Atomic Energy Commission, in the fiscal year 1953, is changing its previous policy of distributing radioisotopes for cancer research and therapy free of production costs. Instead, it will make a charge of 20 per cent of production costs. This change in policy affects only the price, and not the availability of isotopes; production will continue to meet all demands.

The following table shows the prices per millicurie which will be charged, beginning July 1, 1952, for the four most important radioisotopes used in cancer research or therapy, compared with prices for non-cancer use:

	Cancer Use	Noncancer Use
Radioiodine–131	$0.15	$0.75
Radiophosphorus–32	0.22	1.10
Radiogold–198	0.05	0.24
Radiocarbon–14	7.20	36.00

Fellowships and Grants for Scholars in Cancer Research

The Committee on Growth of the National Research Council, acting for the American Cancer Society, is accepting applications for fellowships and for grants for scholars in cancer research.

Fellowship applications must be received by December 10 to be considered for the year 1953–1954. Fellowships ordinarily will begin July 1, 1953, though this date may be varied at the request of the applicant.

Applications for grants for scholars in cancer research should be submitted by an institution on behalf of a candidate prior to January 1, 1953. With these awards, a grant of $6,000 a year for 3 years is paid to the institution.

Communications should be addressed to the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.

Conference on Lung Cancer

An international conference on the "Endemiology of Cancer of the Lung" was held in Louvain, Belgium, July 21 to 24, under the joint auspices of WHO, UNESCO, and the Committee on Geographical Pathology of the International Cancer Research Commission. Called to consider the present status of knowledge on the frequency and etiology of lung cancer, the conference ended by drawing up recommendations for needed future studies. Official representatives to the conference from the United States were Doctors Harold F. Dorn, Cuyler Hammond, Morton Levin, William Smith, Harold L. Stewart, and Paul E. Steiner.

Book Reviews

Leitfaden der Laparokospie und Gastroskopie. By Von Prof. Dr. Med. H. Kalk und Med. Habil. W. Brühl unter Mitwirkung von Dr. Med. W. Burgmann. Stuttgart, Germany: Georg Thieme, Diemershaldenstrasse 47, 1951. Pp. 158.

This book is divided into two sections: one dealing with peritoneoscopy and the other with gastroscopy. The author of the first, H. Kalk, after a brief survey of the historical aspects of peritoneoscopy, describes three types of instruments now in use and discusses the optical principles involved. Kalk's peritoneoscope enables the observer to see the object directly by means of an obliquely directed (135°) lens.

A section on technic follows, with detailed directions on the preparation of the patient, the choice of site for the incision, and the method of introducing the instrument. The writer gives instructions on orientation within the peritoneal cavity and discusses visualization of the abdominal viscera.

Complications consist of hemorrhage due to the puncturing of a blood vessel in the abdominal wall or in the mesentery, and peritonitis. The authors point out that in 2,000 of their own cases there were no deaths, and three fatalities have been reported in Germany. In their series the colon was punctured in four cases and a mesenteric vessel in one.

The authors state that the procedure is to be used only when other diagnostic measures have been exhausted. Unexplained diseases of the peritoneum with ascites, especially when the differential diagnosis lies between tuberculous peritonitis, polyserositis, and metastasis to the peritoneum are indications for this diagnostic procedure. In gynecological conditions tumors of the uterus, ovaries, and tubes, as well as ectopic tubal pregnancy, can be visualized. The main section deals with normal and abnormal findings of the liver, peritoneum, diaphragm, abdominal wall, and the ligamentum teres. The appearance of the liver in the various types of hepatitis and cirrhosis is described in considerable detail and is accompanied by numerous excellent colored illustrations. Disorders of the liver were encountered in two-thirds of the authors' peritoneoscopies. Several pages are devoted to obstructive jaundice, the appearance of the normal and diseased gall bladder, and the extra hepatic biliary ducts.

The second section of the book by W. Brühl deals with gastroscopy, and he follows a similar pattern, with discussion of gastroscopy as an aid to diagnosis and of the types of instruments now in use. In discussing technic the author describes preparation of the patient, anesthesia, and the method of introducing the gastroscope. The reader is instructed in orientation within the stomach, and the blind areas are pointed out. The procedure has become a safe one since the use of Schindler's flexible gastroscope in 1932. However, improvements which have been made in this instrument since then are not mentioned. Shallow ulcers which may be missed roentgenologically can be seen gastroscopically, and the progress of gastric ulcers can be observed more effectively with this modality. Malignant transformation can be detected, and in the case of gastric carcinoma the distinction between polypoid, scirrhous, and ulcerative types can be made. Contra-indications include aneurysms, gastroscopy, esophageal varices, severe kypho-scoliosis, and acute and painful affections of the oral cavity. Complications include esophageal and gastric perforations. Descriptions of gastric ulcers are sufficiently detailed; those of gastric neoplasms could be more complete. Illustrations covering the second section of the book are somewhat meager.

The book does not pretend to be comprehensive in scope, but, as its name implies, it is a very useful handbook.

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Tumors of the Skin. By Joseph J. Eller and William D. Eller. Philadelphia: Lea & Febiger, 1951. Pp. 697.

This is the second edition of a book that has become a standard reference in the field of skin neoplasms. It is chiefly concerned with the diagnosis and treatment of benign and malignant tumors of the skin, but cancer of the lip, mouth, penis, and vulva are included. Also considered are nevi and developmental disturbances of the skin, tumors of infectious origin, precancerous conditions, lymphomas, mycosis fungoides, and Hodgkin's disease.

Histopathologic findings are stressed because of their important bearing on diagnosis and therapeutic management. Fortunately, the authors chose to use standard terminology which is familiar to everyone working in the field of skin tumors. There are many good illustrations of both the clinical and microscopic aspects of the neoplasms considered.

A unique feature of the chapter on the treatment of carcinoma of the skin is the section showing diagrammatic examples of tumors of different types, sizes, shapes, and locations, with a practical discussion of the different methods of treatment which may be used for each lesion. The forms of therapy used by the authors include surgical excision, radium therapy, roentgen therapy, and electrocoagulation. The newly developed method, chemosurgery, is mentioned and credited with having the advantage of microscopic control of excision. However, the authors' unfamiliarity with the technic is indicated by the statement that "a saturated solution of zinc

chloride is swabbed on," whereas the chemical actually is incorporated in a paste vehicle. While some of the diagrams of carcinomas suggest that the neoplasms may extend farther than indicated by the clinical appearance of the lesions, the authors fail to stress that the extent and direction of these outgrowths are often clinically unpredictable, a fact that has been repeatedly demonstrated by means of the microscopically controlled excisions which characterize the chemosurgical method.

The chapter on surgical procedure includes a discussion of plastic repair, including the use of the Reese Dermatome. The principles and technic of roentgen and radium therapy are discussed in the chapter on carcinoma of the skin, and in addition there is a chapter on radiation physics.

The book will continue to be a very useful reference for those interested in the treatment of the considerable variety of lesions which are included under the term "skin tumors."

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Advances in Enzymology, Vol. 14. F. F. NORD (ed.). New York: Interscience Publishers, Inc., 1951. Pp. 570. $9.75.

This volume is unusually rich in material of value to investigators who are pursuing the biochemistry and enzymology of the cancer problem or any other problem that is under attack with the methods of enzymology. Few of the volumes in this series have collected at one time the efforts of such outstanding authorities or topics of such broad interest. In addition there is a juxtaposition of reviews that brings together not just one expert opinion but two or even three on a given topic, with a result that is both stimulating and authoritative. Thus certain aspects of photosynthesis are discussed by Robert Hill, and again by M. F. Utter and H. G. Wood; enzyme substrate interaction is discussed from three different viewpoints by Britton Chance, by Emil Smith, and by David Nachmansohn and Irwin B. Wilson, each of whom is outstanding in the fields which he discusses; the present status of starch chemistry is reviewed by Kurt H. Meyer and G. C. Gibbons, while the enzymes of starch degradation and synthesis are discussed by Peter Bernfeld. The only topics not given the many-sided approach are biological methylation by Frederick Challenger and the reaction of borate with substances of biological interest by C. A. Zittle. Since the latter is largely concerned with reactions involving sugars and polysaccharides having adjacent hydroxyl groups, it supplements the reviews on starch chemistry and enzymology. The review by Challenger is systematically done, but even with 211 references it could not possibly contain the latest reports in a rapidly moving field.

Although none of the papers is directly concerned with cancer metabolism per se, the discussions on methylation reactions and carbon dioxide assimilation would be required reading for investigators in this field, while those interested in chemotherapy will benefit by the discussions on enzyme-substrate relationships. The over-all scope of the chapter by Utter and Wood and its fundamental nature are indicated by the following sentences from their introduction: "The ability of autotrophic forms of life to bring about a total synthesis of their cellular material from carbon dioxide and other inorganic materials is one of the most baffling problems in biochemistry. . . . Stated in the simplest way, autotrophic synthesis becomes a problem of understanding the fixation of carbon dioxide in adjacent carbon atoms of a compound." "The problem of understanding the reactions whereby the carbohydrates, fats, and proteins are built from CO_2 does not appear as insurmountable as it did a few years ago." These are brave words, and if we can hope to understand the metabolism of the autotrophs, surely the metabolism of the higher forms can be encompassed, for, as Utter and Wood state, "It might be expected that in the spectrum of living forms there would be biochemical gradations from the completely autotrophic forms to the typical heterotrophs. This has proved to be the case." However, cancer investigators will take small comfort from the staggering amount of effort required to understand how carbon dioxide is combined with a three-carbon compound to yield a four-carbon dicarboxylic acid. If this reaction can be the subject of 75 references and 35 pages of discussion, with the issue still not understood, how many confused issues must arise before the biochemical mechanisms of growth control will be clarified? Utter and Wood perform a real service in their careful organization of material, frequent summaries of sub-sections, and critical exercise of judgement. The latter is noteworthy in the section on the evaluation of C^{14} methods, and the over-all impression given by the chapter is a justification of the cautious optimism expressed in the introduction. The other chapters are all of great intrinsic interest and together make this one of the finest volumes in this series.

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Advances in Genetics, Vol. IV. M. DEMEREC (ed.). New York: Academic Press, Inc., 1951. Pp. 343.

The high standard of excellence set in the previous three volumes of this series is maintained in this publication. The first three of the nine separate articles comprising the volume are most likely to interest workers in the field of cancer research. Gluecksohn-Waelsch discusses the physiological genetics of the mouse in a review of the development of a number of mutant characters. Although very little new material is included in the article and it is limited in scope, the oncologist will find his tumor stocks to bear some of the mutants which are included. Hadorn gives a well organized account of the action of lethal factors on the development of Drosophila from the standpoint of specificity as to phase

of development, with a section on patterns of damage due to pleiotropic action of mutants. Physiological as well as morphological effects of lethals are included, along with a discussion of the influence of environment, combined genic action, phenocopies,[1] and information obtained from transplantation experiments. The cytological and genetical relationships of euchromatin and heterochromatin in mitotic and salivary gland chromosomes and the present status of position effect are given by Hannah. A number of investigators will probably not regard some of her statements justified on the basis of current laboratory evidence, but her presentation is extensive and interesting. The current quest for information concerning the mode of genic action is reflected in these three reviews. Advances in the fields reported should result in clearer concepts about susceptibility to cancer.

Other articles include development of the thesis that duplications may be significant in evolution by Stephens, with the conclusion that divergence of duplicates has not been proved; a scholarly presentation of the cytogenetics of Orthopteroid insects by M. J. D. White; and Hirschberg's translation of an account of the chromosomes in the vertebrates by Matthey, which is very good but somewhat marred by an abbreviated bibliography—the reader being referred to the author's book for its completion. The remaining titles concerning the genetics of Coffea, rice, and cotton are unlikely to be of more than passing interest to the worker in the field of cancer. Inability to meet the interests of every reader is of course inherent in any volume of this type. The book is pleasing in format and type, the illustrations are adequate, and it is reasonable in price. Continued annual publication of the series is quite desirable.

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Cold Spring Harbor Symposia on Quantitative Biology, Vol. XV. Origin and Evolution of Man. Lancaster: Science Press, 1951. Pp. 425.

Syllabus of Human Neoplasms. By R. M. MULLIGAN. Philadelphia: Lea & Febiger, 1951. Pp. 317.

Lehrbuch der Allgemeinen Pathologie und der Pathologischen Anatomie. By HERWIG HAMPERL. Berlin, Gottingen, & Heidelberg: Springer-Verlag, 1950. Pp. 788.

Tumors of the Adrenal. By HOWARD T. KARSNER. Washington, D.C.: Armed Forces Institute of Pathology, 1950. $1.00.

Manual of Tumor Nomenclature and Coding. New York: American Cancer Society, 1951. Pp. 119. $2.50.

Über die Rontgenologischen Darstellungsmöglichkeiten des weiblichen Genitalapparates mit hilfe von Jodol und Jodsol. (The Roentgenological Visualization of the Female Reproductive Organs with the Aid of Iodized Oils.) By J. ERBSLÖH. In German. Stuttgart: Georg Thieme Verlag, 1951. Pp. 74.

Announcement

Theodor Steinkopff, publishers of Dresden, Germany, announce the publication of the journal *Zeitschrift für Altersforschung*. The journal will publish original articles, book reviews, and abstracts on all medical problems concerned with aging. It will appear quarterly, and Dr. M. Bürger of Leipzig is the editor.

This journal was first published in 1939 with Drs. E. Abderhalden and M. Bürger as editors, but it was discontinued in 1944 after the first number of Volume 5.